UNIVERSIDAD PABLO DE OLAVIDE

Doctorado en Ciencias de la Actividad Física y del Deporte

TESIS DOCTORAL

Efecto de la Cotinina y Aceite de Krill sobre el tejido neuroglial, actividad neurocognitiva y comportamiento, en ratones C57BL/6 expuestos a estrés.

TESIS PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

CRISTHIAN ALEJANDRO MENDOZA SEPÚLVEDA

DIRECTORA

DRA. RAQUEL PÉREZ ORDÁS

SEVILLA, ABRIL 2018

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DOCTORANDO

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- Investigating Cotinine as a Therapy for Post-traumatic Stress Disorder
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SAN SEBASTIAN

Concepción, 2 de Abril de 2018.

Dra. Raquel Pérez Órdas

Programa de Doctorado en Ciencias
de la Actividad Física y del Deporte
Universidad Pablo de Olavide
Sevilla, España

Estimada Dra. Raquel Pérez Órdas

Le escribo para comunicarle que el Profesor Cristhian Mendoza Sepúlveda Rut:14281296-7, a desarrollado su tesis doctoral en el marco del Proyecto FONDECYT (Fondo Nacional de Ciencia y Tecnología) regular #1150194, del Gobierno de Chile.

Este proyecto ha permitido la publicación en revistas con comité editorial de alto impacto (cuatro manuscritos y un quinto en evaluación). Además, estos resultados han dado lugar a una patente internacional recientemente enviada a la oficina de patentes en EEUU.

El Sr. Mendoza, continuará sus investigaciones prontamente para desarrollar un trabajo relacionado con los efectos del estrés sobre la atrofia muscular inducida por el estrés de inmovilización.

Tengo la certeza que Mr. Cristhian Mendoza contribuirá grandemente al desarrollo del posgrado y la investigación en nuestra Universidad y será un neo de colaboración entre nuestras Universidades.

Sin otro particular le saluda atentamente.

Valentina Echeverría Moran, BQ. MSc. PhD.
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PUBLICACIONES Y COMUNICACIONES DEL AUTOR RELACIONADOS CON LA TESIS

Artículos publicados en revistas científicas de impacto (JCR)

- **Mendoza C.**, Barreto G., Ávila-Rodríguez M., Echeverría V. (2016). Role of neuroinflammation and sex hormones in war-related PTSD. J. Molecular and Cellular Endocrinology. 434: 266-277

- Pérez-Urrutia N., **Mendoza C.**, Álvarez-Ricartes N., Oliveros-Matus P., Echeverría F., Grizzell J.A., Barreto G., Iarkov A., Echeverría V. (2017). Intranasal cotinine improves memory, and reduces depressive-like behavior, and GFAP + cells loss induced by restraint stress in mice. J. Experimental Neurology. 295: 211-221

- Álvarez-Ricartes N., Oliveros-Matus P., **Mendoza C.**, Pérez-Urrutia N., Echeverría F., Iarkov A., Barreto G., Echeverría V. (2018). Intranasal Cotinine Plus Krill Oil Facilitates Fear Extinction, Decreases Depressive-Like Behavior, and Increases Hippocampal Calcineurin A Levels in Mice. J. Molecular Neurobiology. [Epub ahead of print].

- **Mendoza C.**, Barreto G., Iarkov A., Tarasov V.V., Aliev G., Echeverría V. (2018). Cotinine: A Therapy for Memory Extinction in Post-Traumatic Stress Disorder. J. Molecular Neurobiology. [Epub ahead of print].

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- **Mendoza C.**, Pérez-Urrutia N., Álvarez-Ricartes N., Barreto G., Pérez-Ordás R., Iarkov A., Echeverría V. (2018). Cotinine plus Krill Oil decrease depressive behavior, and increased astrocytes survival in the hippocampus of mice subjected to restraint stress.

Congresos

1. **Society for Neuroscience. Neuroscience 2016 Annual Meeting. San Diego, California, EEUU. 12 noviembre de 2016**

Presentation: Poster

Title: “Posttreatment with cotinine alleviates symptoms in a mouse model of chronic stress”.

Authors: Iarkov A., Echeverría V., Pérez-Urrutia N., **Mendoza C.**, Álvarez-Ricartes N., Echeverría F.

Abstract:
Several psychiatric symptoms such as depression, anxiety, hyperactivity and cognitive impairment appears a result of chronic stress and model PTSD symptoms. These symptoms are accompanied by hormonal changes, such as excessive activation hypothalamus pituitary-adrenal axis and the deregulation of several neurotransmitter signaling pathways, such as the sympathetic and serotonergic systems. Chronic or traumatic stress induces pathological changes in several brain regions of the fear network, including medial prefrontal cortex, medial temporal lobe system, the amygdala and hippocampus. Cotinine, considered a positive allosteric modulator of the alpha 7 nicotinic acetylcholine receptor (nAChR), when infused directly in to the hippocampus, enhanced fear extinction in rats in a manner dependent on the activity of the nAChRs. Cotinine also stimulated downstream effectors of the alpha 7nAChR including the protein kinase B-Glycogen synthase kinase 3beta pathway and the extracellular signal-regulated kinases. Our hypothesis is that cotinine by these mechanisms can relieve these symptoms as a posttreatment. Thus, the positive effects of cotinine as a Posttreatment on anxiety, visual recognition memory, depressive-like behavior that resulted from prolonged restrain stress. Mice were restrained 6 hours/day for 21 days. Treatment started after immobilization and extended for 14 days. Cotinine was administered via gavage (0.5 mg/kg in PBS). The impact of immobilization stress on mice´s behavior was evaluated with battery of behavioral tests (open field, Porsolt´s test, novel object recognition, light dark box test, and elevated plus maze). Our findings suggest that cotinine can be used as a treatment option to alleviate symptoms derived of chronic stress.

RESUMEN

La exposición a diversas formas de estrés intenso o crónico, induce importantes alteraciones neuroquímicas, morfológicas y funcionales en el tejido cerebral, lo que genera finalmente, en el individuo, déficits cognitivos y un comportamiento depresivo. Estas alteraciones están asociadas a procesos neuroinflamatorios y neurodegenerativos, que afectan tanto a neuronas como al tejido glial. Es crucial destacar las distintas capacidades funcionales de las células astrocíticas, en especial en regiones claves para el aprendizaje, memoria y respuestas emocionales, como el hipocampo y la corteza prefrontal. También es importante comprender cómo la disfunción astrocítica, está relacionada a la pérdida de memoria, trastorno depresivo mayor y otras condiciones neuropsiquiátricas.

Considerando como base lo anterior, se estableció el siguiente objetivo general para el desarrollo de esta tesis:

- Determinar los efectos que produce la suplementación con cotinina y/o aceite de Krill, como cotratamiento o post tratamiento, sobre el tejido neuroglial, comportamiento, estado anímico y la memoria, en ratones C57BL/6, expuestos a estrés.

Materiales y métodos

a) Animales: Se utilizaron ratones (cepa C57BL/6) facilitados por la Universidad de Chile. Mantenidos en un ambiente controlado (ciclo 12h:12h luz/oscuridad, temperatura de 22°C.) y acceso libre a alimento y agua (ad libitum). El manejo y cuidado de los animales se realizó de acuerdo a la “Guide for the care and use of Laboratory Animals” del National Institute of Health (USA).

b) Drogas y reactivos: cotinina ((5S)-methyl-5-(3-pyridyl)-pyrrolidin-2-one) fue obtenido de Sigma-Aldrich (St. Louis, MO, USA). Aceite de krill obtenido de softgels capsules of krill oil omega-3 of Walgreens (Superba, USA). Las cápsulas contienen 300 mg de aceite de krill (90 mg de ácidos grasos omega-3, 50 mg EPA, 24 mg

DHA, 130 mg de fosfolípidos). El fabricante no provee información sobre el contenido de astaxantina.

c) Administración de drogas: se utilizó vía intranasal (IN) y gavage (oral), según cada protocolo.

d) Metodología: en general se utilizaron dos métodos de estrés (restricción de la movilidad y condicionamiento de miedo contextual). Los animales se agruparon para estudiar los efectos pre y post estrés, cotratamiento o post tratamiento con las respectivas drogas versus vehículo (solución salina), según cada estudio.
Diseño experimental específico, descrito en cada publicación, según los objetivos propuestos.

e) Estudios de comportamiento: test para actividad locomotora o de campo abierto (OF); test para memoria de trabajo o reconocimiento de objeto nuevo (NOR); test para evaluar comportamiento depresivo o test de nado forzado (FST), y para la ansiedad, test del laberinto elevado (EPM).
En el caso del condicionamiento de miedo en los animales, se realizaron los test de retención de memoria de miedo y extinción de miedo contextual.
El comportamiento animal fue grabado y analizado usando el Any-maze software (Stoelting Co, Illinois, USA).

f) Análisis morfológicos e inmunohistoquímica: se realizó preparación histológica de tejidos (corteza prefrontal e hipocampo), análisis inmunohistoquímico de células GFAP+ y análisis cuantitativo fractal (área celular, área de arborización y lacunaridad). Información in extenso en publicaciones respectivas.

g) Análisis bioquímico: análisis de proteína fosfatasa calcineurina en el hipocampo y corteza prefrontal. Se utilizó anticuerpo policlonal de conejo para calcineurina (PP2B) que fue obtenida de Cell Signaling Technology.

h) Análisis estadístico: descrito en cada publicación.

Resumiendo, algunas de las conclusiones más importantes de esta tesis son:

1. La cotinina protege las células astrocíticas GFAP+, restaurando la supervivencia neuronal y la plasticidad después del estrés.
2. La cotinina, administrada vía intranasal, restaura las capacidades cognitivas y previene o mejora la conducta depresiva inducida por el estrés.
3. La cotinina intranasal sola o en combinación con aceite de krill, facilita la extinción de la memoria de miedo contextual y disminuye el comportamiento depresivo, mediante un mecanismo que involucra la estimulación de la expresión de la calcineurina, después del condicionamiento de miedo en ratones.
4. La cotinina intranasal más aceite de krill, reduce los síntomas depresivos derivados de recuerdos traumáticos asociativos inducidos por el comportamiento de miedo, en forma más efectiva que la cotinina administrada como terapia única.
5. La mezcla de cotinina más aceite de krill, evitó el comportamiento depresivo, la alteración de la memoria y los trastornos en los astrocitos, inducidos por la restricción prolongada del movimiento.

ABREVIATURAS

SNC: sistema nervioso central

PFC: corteza prefrontal

HPA: eje hipotálamo-pituitaria-adrenal

NAChR: receptor nicotínico de acetilcolina

α7nChR: receptor nicotínico de acetilcolina alfa 7

GFAP: proteína ácida fibrilar glial

BDNF: factor neurotrófico derivado del cerebro

DHA: ácido docosahexaenoico

EPA: ácido eicosapentaenoico

MDD: trastorno depresivo mayor

PD: enfermedad de Parkinson

AD: enfermedad de Alzheimer

NFκB: factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas

TNF-α: factor de necrosis tumoral alfa

PUFA: ácido graso poliinsaturado

ω-3: ácido graso omega 3

KO: aceite de Krill

AXT: astaxantina

PTSD: trastorno por estrés postraumático

DG: giro dentado del hipocampo

LTP: potenciación a largo plazo

LTD: depresión a largo plazo

GABA: ácido gamma-aminobutírico

NMDA: ácido N-metil-D-aspártico

SOD: superóxido dismutasa

EAAT2: transportador de glutamato de alta afinidad o transportador de aminoácidos excitatorios

ATP: adenosín trifosfato

ATX: antioxidante

PAM: modulador alostérico positivo

TAU: proteína Tau.

ÍNDICE DE CONTENIDOS

I. JUSTIFICACIÓN DEL ESTUDIO

El término estrés define todas las respuestas fisiológicas y/o psicológicas a los eventos que requieren un ajuste conductual para superarlos.

El estrés agudo incluye mecanismos adaptativos necesarios para la supervivencia, mientras que el estrés crónico induce la sobreactivación y la disfunción de los sistemas activados por el estrés, lo que provoca daño cerebral y un comportamiento depresivo[1,2].

El estrés por restricción afecta tanto la memoria espacial dependiente del hipocampo[3,4] como la potenciación a largo plazo del hipocampo[5]. Tales efectos se han asociado a la retracción de dendritas apicales, así como la pérdida de sinapsis en la subregión CA3 del hipocampo. La explicación propuesta, entre otras, es que estos cambios pueden estar asociados con la liberación desregulada de glutamato y disfunción del receptor de NMDA[6,7,8].

Es importante también conocer los efectos deletéreos que produce la inmovilización o restricción del movimiento, como evento estresante, no sólo en el ámbito cardiorrespiratorio, artromuscular o metabólico, sino también en el aspecto neurocognitivo y estado anímico; más aún, en individuos que han tenido una larga estadía en unidades de cuidado intensivo, pacientes postrados, o bien, personas cursando períodos de estrés que les impide mantener una actividad física regular.

El estrés agudo tiene muchos efectos beneficiosos, sin embargo, el estrés crónico contribuye a una variedad de problemas de salud como ansiedad, depresión, problemas gastrointestinales, enfermedades cardiacas, desórdenes del sueño y obesidad, entre otros. Mientras que el estrés agudo puede ser beneficioso para reclutar respuestas adaptativas para hacer frente a la situación estresante, el estrés prolongado puede resultar en una mala adaptación que puede ser un factor de riesgo de numerosas enfermedades mentales afectivas. Los estudios en animales han revelado una reducción de las neuronas del hipocampo en la exposición al estrés crónico[9, 10, 11].

Los estudios clínicos han demostrado que los individuos sometidos a estrés durante largo tiempo, muestran un volumen hipocampal reducido, además de la degeneración de otras regiones del cerebro límbico. Se ha reportado reducción del volumen hipocampal en individuos con trastorno depresivo recurrente y trastorno de estrés postraumático[12, 13, 14].

Hay evidencia creciente, de estudios en animales, que el estrés crónico tiene efectos sobre la morfología de las células gliales, el metabolismo y la función en la corteza prefrontal (PFC) y posiblemente también en el hipocampo.

Tradicionalmente, por muchos años, fue considerado que la glía tenía una existencia pasiva en el sistema nervioso central, sólo como soporte estructural y nutricional de las neuronas. Sin embargo, recientes estudios han demostrado que la glía puede regular la formación sináptica, controlar la eficacia sináptica y participar en el procesamiento de información por su interacción con las neuronas[15,16, 17]. El procesamiento de información se regula a través de incrementos en los niveles de Ca^{2+} citoplasmáticos neuronales y se asocia con cambios en la fuerza sináptica en sinapsis adyacentes. Esta regulación sináptica se debe en parte a la capacidad de los astrocitos para regular la actividad de las neuronas, a través de la liberación de ATP [18], o la liberación de glutamato[19, 20, 21], y la posterior activación de los receptores de glutamato en las células neuronales y gliales.

El papel dinámico de la glía en la regulación de la eficacia sináptica depende de la cercanía física de los procesos astrocíticos con las neuronas y la subsiguiente modulación astrocítica de los receptores neuronales, que regulan tanto la liberación como la respuesta al neurotransmisor. La formación de procesos astrocíticos estables en respuesta a las neuronas requiere la presencia de un filamento intermedio astrocítico, la GFAP[22].

La disminución de la capacidad para eliminar el glutamato extracelular como resultado de la alteración de la captación y el metabolismo de las células gliales, combinada con los cambios inducidos por el estrés en la liberación de glutamato y la función del receptor de glutamato, podría proporcionar un mecanismo fisiopatológico que conduce a muchos de los cambios cerebrales (neuroglia) en individuos con trastornos psiquiátricos asociados al estrés, tales como, cambios en el estado de ánimo y los trastornos de ansiedad[23].

La restricción del movimiento durante el envejecimiento y en enfermedades agudas y crónicas, induce cambios en la estructura y función de los astrocitos de la zona del hipocampo, la cual es una de las estructuras más vulnerables del cerebro al estrés oxidativo. Estos cambios incluyen una alteración en la expresión de marcadores típicos de astrocitos tales como la proteína glial fibrilar acídica (glial fibrillary acidic protein, GFAP), Vimentina y de otros marcadores de citoesqueleto incluyendo la actina, y de transportadores como GLAST y GLT-1 (EAAT2) y enzimas del metabolismo de glutamato (glutamato sintasa)[15, 24, 25].

Los astrocitos cobran mucha importancia durante el estrés oxidativo al desencadenar la respuesta inflamatoria aumentando los niveles de citokinas proinflamatorias como TNFα, IL-1β, IL-6, IL-18 y de RNA mensajero de COX-2, cuyos niveles se ven alterados durante los procesos inflamatorios[26, 27]. Los astrocitos también se ven afectados tras la liberación de factores neurotróficos, incluyendo GDNF, BDNF, S100B y TGF-β[28, 29].

La evidencia experimental hasta ahora, refuerza el papel del estudio de los astrocitos del hipocampo como un objetivo para la comprensión de los mecanismos involucrados en el estrés por inmovilización, así como otras enfermedades neurológicas, proporcionando una herramienta innovadora para los estudios de astrocitos en el cerebro fisiológico y patológico[27].

Conociendo el papel esencial de los astrocitos en la funcionalidad cerebral y neuroprotección, esta capacidad se va perdiendo con el envejecimiento y en procesos de estrés causados por distintos estímulos aversivos; lo que ha permitido asociar este declive con enfermedades neurológicas[30, 31, 32].

El GFAP está involucrado en el mantenimiento de la citoarquitectura celular (SNC), estabilidad mecánica y función sináptica, propiedades que se ven afectadas notablemente en el envejecimiento[33]. Por ejemplo, la disminución de la expresión de GFAP, que refleja la degeneración astroglial, se ha encontrado en estados tempranos de muchas enfermedades neurodegenerativas. Diversos estudios han reportado disminución de GFAP y una reducción del número de células positivas GFAP en el hipocampo, después de 5 semanas de estrés. Hughes et al, sugieren que GFAP juega un rol clave en la inducción de GLT-1 en hipocampo[34].

Similarmente, la disminución del receptor GLT-1 ha sido observada en numerosos estudios de enfermedades neurodegenerativas, como la enfermedad de Alzheimer, donde los astrocitos del hipocampo resultan ideales para estudiar cambios inducidos en el cerebro por esta enfermedad[35]. Reducciones o disfunciones de GLT1 se han documentado en varios trastornos neurológicos, incluyendo accidente cerebrovascular, la enfermedad de Alzheimer (Alzheimer's Disease, AD) y la esclerosis lateral amiotrófica. Estos hallazgos destacan la importancia de GLT-1 en astrocitos para la función cerebral normal[36, 37, 38].

El glutamato regula la transmisión sináptica y plasticidad por la activación de receptores de glutamato ionotrópicos (AMPA y NMDA) y metabotrópicos (mGluR 1-8). El número y estabilidad de estos receptores en la membrana sináptica es un importante factor en determinar la eficacia de la sinapsis excitatoria.

El glutamato es aclarado desde el espacio extracelular vía receptores de alta afinidad (EAAT) localizados en la glía. En las células gliales, el glutamato es convertido en glutamina por la glutamina sintetasa. La glutamina es entonces transportada de nuevo a la neurona glutamatérgica, donde es hidrolizada en glutamato por la glutaminasa. Debido a la falta de enzimas degradativas en la sinapsis, la absorción por las EAAT es el principal mecanismo a través del cual se termina la acción del glutamato extracelular[39,40].

El transportador EAAT2 (GLT-1 en roedores) es el transportador de glutamato astroglial predominante en el hipocampo y corteza prefrontal; es responsable del

mayor consumo de glutamato extracelular (90%), introduciéndolo al astrocito donde es convertido en glutamina no tóxica, por la enzima glutamina sintetasa. Estudios en personas con depresión mayor[40] y modelos animales de depresión[41, 42, 43] han revelado alteraciones en la expresión de los niveles de GLT-1.

Sin duda, es fundamental la conservación de la estructura y función de la astroglía, para el mantenimiento de la homeostasis cerebral, la actividad y fuerza sináptica, el equilibrio de neurotransmisores; más aún, cuando nuestro organismo se ve enfrentado a situaciones de estrés de distinta índole y grado.

La evidencia actual indica claramente que la neuroinflamación desempeña un papel importante en la etiología y el desarrollo de varios trastornos neurológicos, incluyendo enfermedades neurodegenerativas como AD y la enfermedad de Parkinson (Parkinson's Disease, PD) y las condiciones psiquiátricas, incluyendo la depresión mayor, los trastornos bipolares, el trastorno por estrés post traumático y como efecto de períodos prolongados de inmovilización, como ocurre en pacientes postrados o internados en unidades de cuidado intensivo y que conducen a disminución franca de la funcionalidad e independencia de los individuos.

La cotinina es el principal metabolito de la nicotina, actuando como modulador alostérico positivo del receptor nicotínico de la acetilcolina (α7nAChR) y estimulando los efectores del α7nAChR, incluyendo la Akt/GSK3β, ERK, mTOR, promoviendo así la supervivencia neuronal y la plasticidad sináptica, disminuyendo la ansiedad y el comportamiento depresivo[44, 45].

La modulación positiva de los α7 nAChRs con cotinina y otros compuestos similares puede reducir claramente la neuroinflamación, así como aumentar la resiliencia de las células cerebrales a las lesiones tóxicas y prevenir los cambios en el estado de ánimo y las habilidades cognitivas que se ven afectados en muchos trastornos neurológicos (ver estudios previos).

Se ha demostrado que la cotinina evita la pérdida de la memoria de referencia y de trabajo en un modelo de ratón de AD[46]. El tratamiento con cotinina también redujo el número y tamaño de las placas amiloides[47]. Un estudio reciente investigó el efecto del pre y post tratamiento con cotinina sobre la estabilidad de la memoria del miedo contextual después de la reexposición repetitiva o única al contexto. Mostrando que la cotinina aceleró la extinción y redujo la estabilidad de la memoria contextual del miedo[48].

En consonancia con estos hallazgos, en el estudio realizado por Chen et al. (2005) la mejora de la extinción del miedo contextual, inducida por el tratamiento de cotinina en ratones C57BL/6, se correlacionó positivamente con un aumento en los niveles de la forma activa de ERK1 / 2 (fosfo-ERKs) en el hipocampo[49].

Una hipótesis propuesta para explicar los efectos beneficiosos de la cotinina sobre la cognición es la teoría de la modulación positiva de la población específica de los α7nAChRs. Ésta, especula que la modulación de cotinina en los α7nAChRs expresados en neuronas GABAérgicas inhibidoras del hipocampo, puede resultar en la activación de receptores excitatorios de glutamato que median los cambios de plasticidad sináptica requeridos para la memoria[50, 51].

En ratones, el tratamiento a largo plazo con cotinina indujo la activación de la vía de señalización de Akt-/-GSK3β, que es activada por el α7nAChR, tanto en el hipocampo como en la corteza[46].

En vista de lo anterior, para el desarrollo de esta tesis, se consideró el trabajo con modelos animales de estrés, para evaluar los cambios en el comportamiento, estado anímico, el procesamiento cognitivo y muy importante, para observar los cambios o modificaciones histo-morfológicas que experimentan los astrocitos y su expresión de proteínas del citoesqueleto como GFAP. Considerando el concepto que el estrés (en este caso mecánico) logra producir un efecto psicocognitivo, evidenciado con las evaluaciones de comportamiento, utilizadas y validadas internacionalmente. A su vez, para estudiar los eventos celulares se utilizó el procesamiento histológico y análisis inmunohistoquímico.

Los protocolos de inmovilización que se utilizaron (estrés por restricción o modelo de PTSD), como paradigma de estrés psicológico, causan neuroinflamación y neurodegeneración a nivel hipocampal, siendo un modelo experimental muy utilizado para analizar los cambios inducidos por el estrés en el cerebro.

De acuerdo a la evidencia existente, respecto a la relación entre la neuroinflamación y neurodegeneración, se hace imprescindible el hallazgo de suplementos que permitan revertir este proceso, entre ellas el consumo de ácidos grasos poliinsaturados, n-3 PUFA, como DHA y EPA. El DHA atraviesa la barrera hematoencefálica y se concentra abundantemente en el cerebro adulto, especialmente en zonas asociadas a la memoria y aprendizaje. En roedores, representa aproximadamente el 20% de lípidos totales en el cerebro[52]. La suplementación con dicho ácido graso se ha correlacionado, en seres humanos, con menor riesgo de deterioro cognitivo, particularmente lo asociado a fluidez verbal[53] e inversamente con la inflamación del hipocampo[54]; afectando la diferenciación neuronal mediante la promoción del crecimiento de neuritas en esta estructura cerebral, pudiendo tener el potencial de aumentar la neurogénesis[55, 56, 57].

El DHA es un ácido graso poliinsaturado, que se encuentra en diferentes productos de origen animal, entre ellos el Krill antártico (Euphausia superba), crustáceo marino con elevado contenido de ácidos grasos y astaxantina, un carotenoide con gran

capacidad antioxidante[58]. Al administrarse en modelo animal, durante 7 semanas, se ha observado un aumento en el proceso de aprendizaje, con efectos antidepresivos[59]; por otra parte, la suplementación durante por lo menos 30 días, provoca una reducción de la inmovilidad en pruebas de natación forzadas, cuyo objetivo es evaluar el nivel de depresión de los animales[60].

El efecto de los ácidos grasos poliinsaturados, particularmente DHA y EPA sobre el sistema nervioso podría generarse mediante la regulación del segundo mensajero el AMPc y la expresión de factor neurotrófico derivado del cerebro (brain-derived neurotrophic factor, BDNF)[61]. Hallazgos emergentes sugieren que este factor tiene un papel importante en la regulación de la homeostasis energética, mediando los efectos beneficiosos del ejercicio vigoroso y ayuno en la cognición, el estado de ánimo, la función cardiovascular y el metabolismo[62].

Por todo lo anteriormente expuesto, esta investigación está focalizada en dilucidar los efectos de los fármacos cotinina y aceite de krill (solos o mezclados) sobre los procesos cognitivos, memoria, ansiedad, depresión e histoestructura astrocítica en el hipocampo, en animales de estudio bajo estrés. Estos fármacos poseen capacidades que permiten restablecer la función de los astrocitos y neuronas. Se les asocia con neurogénesis, disminución del estado depresivo/ansioso, sinaptogénesis, efecto antiinflamatorio, mejora de la extinción de la memoria de miedo, aprendizaje y neuroplasticidad, entre otras.

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II. PREGUNTAS DE INVESTIGACIÓN

¿Cuál es el real efecto del estrés sobre la memoria y el comportamiento en ratones C57BL/6, modelo restricción del movimiento?

¿Qué modificaciones histológicas o inmunohistoquímicas produce el estrés sobre la población astrocítica en el hipocampo de ratones C57BL/6?

¿Podrá la suplementación con cotinina y/o aceite de Krill, de manera individual o mezclados, atenuar los efectos deletéreos del estrés sobre la cognición, estado anímico, o reactividad del GFAP en astrocitos hipocampales, en ratones C57BL/6?

¿Presentará mejores resultados la suplementación con cotinina y/o aceite de Krill (de manera individual o mezclados) en modalidad de cotratamiento o posttratamiento, en animales expuestos a estrés?

III. OBJETIVOS

A. General

1. Determinar los efectos que produce la suplementación con cotinina y/o aceite de Krill, como cotratamiento o post tratamiento, sobre el tejido neuroglial, comportamiento, estado anímico y la memoria, en ratones C57BL/6 expuestos a estrés.

B. Específicos

1. Evaluar el comportamiento animal pre y post exposición a estrés (actividad locomotora, memoria de trabajo, comportamiento depresivo y ansiedad). (paper N°2, 3 y 5 en revisión).

2. Evaluar la actividad locomotora, memoria de trabajo, comportamiento depresivo y ansiedad, en ratones suplementados con cotinina (intranasal, post tratamiento), expuestos a estrés por inmovilización. (paper N°2).

3. Evaluar la actividad locomotora, memoria de trabajo, comportamiento depresivo y ansiedad, en ratones estresados, suplementados con cotinina, aceite de krill o la mezcla de ambos, como cotratamiento. (paper N°5 en revisión).

4. Evaluar estado depresivo, consolidación y extinción del miedo en ratones, modelo condicionamiento de miedo contextual, suplementados con cotinina, aceite de krill o la mezcla de ambos, como cotratamiento. (paper N°3).

5. Describir los cambios morfológicos que ocurren en los astrocitos del hipocampo, pre y post estrés. (paper N°2 y 5 en revisión).

6. Describir los cambios morfológicos que ocurren en los astrocitos del hipocampo suplementados con cotinina, aceite de krill o la mezcla de ambos. (paper N°2 y 5 en revisión), en animales expuestos a estrés por restricción de la movilidad.

7. Evaluar el efecto de la cotinina, aceite de krill o la mezcla de ambos, en los marcadores de células neurogliales hipocampales (GFAP+), en animales expuestos a estrés crónico (paper N°2 y 5 en revisión).

8. Comprender el rol de la neuroinflamación en PTSD (paper N°1, Review)

9. Revisar la evidencia actual que respalda el uso de cotinina en PTSD (paper N°4, Review)

IV. MARCO TEÓRICO

A. INTRODUCCIÓN

Si en los siglos pasados, las epidemias de peste negra, cólera y otras enfermedades infectocontagiosas diezmaban a la población mundial, a partir del siglo XX y con la introducción de antibióticos y vacunas, las enfermedades crónicas no transmisibles han llegado a ser el verdadero problema de salud pública; al menos en países desarrollados y en vías de desarrollo.

Entre estas enfermedades, no sólo encontramos a la Diabetes Mellitus, la Hipertensión Arterial y el Asma, sino también a una diversidad de enfermedades mentales y trastornos psiquiátricos que afectan a un porcentaje, cada vez mayor, de la población mundial.

La OMS define salud mental como un estado de bienestar en el cual un individuo es consciente de sus propias capacidades, puede afrontar las tensiones normales de la vida, puede trabajar en forma productiva y fructífera y es capaz de hacer una contribución a su comunidad.

El Plan de Acción Integral sobre Salud Mental 2013-2020 de la OMS, reconoce que la salud mental es un elemento esencial para el bienestar de todas las personas y entre sus objetivos encontramos el fortalecer los sistemas de información, los datos científicos y las investigaciones sobre salud mental.

En esta tesis se buscó no sólo obtener una mayor comprensión de los efectos del estrés sobre el estado neurocognitivo, comportamiento y población astroglial, en un modelo animal de estrés crónico, sino también descubrir los efectos específicos de los fármacos Cotinina y Aceite de Krill, sobre estas variables, y contribuir a obtener nuevos productos que apoyen el tratamiento en enfermedades mentales.

B. SISTEMA NERVIOSO

Sería un objetivo inalcanzable, detallar todas las capacidades, funciones y actividades que desarrolla nuestro sistema nervioso. Pero, sin duda, una de sus especialidades, es su gran capacidad para almacenar información en forma de memoria. Esa información retenida puede ser recuperada en un momento determinado, para así responder ante situaciones o eventos ambientales particulares. Este proceso de formación de recuerdos y de especialización funcional en el cerebro, durante el desarrollo, está mediado por su plasticidad estructural y funcional [1]. Es importante señalar que nuestro cerebro posee una plasticidad notable, capaz de crear y eliminar sinapsis rápidamente, así como de alterar los circuitos funcionales en la adaptación y el aprendizaje.

Es menester saber que, durante bastante tiempo, el tejido neuronal fue la base de estudio y explicación para muchos procesos psicológicos y trastornos neurológicos; sin embargo, hoy la neuroglia comienza a considerarse como un elemento central en la neurofisiología y neuropatología [2]. Las investigaciones que se han ejecutado para responder a todas las incertidumbres sobre la plasticidad, a nivel del sistema nervioso central, se han focalizado en el tejido neuronal; no obstante, debemos prestar gran atención a la injerencia que tiene el tejido neuroglial, particularmente los astrocitos, en el control y funcionamiento de este mecanismo [1]. Los astrocitos son, en parte, responsables de regular la formación de sinapsis y la actividad sináptica; existe una gran posibilidad de que su actividad homeostática desempeñe un papel integral en la plasticidad y el aprendizaje.

1. Sinapsis y Espinas Dendríticas

Las sinapsis son estructuras especializadas entre neuronas que permiten la comunicación neuronal. Los neurotransmisores se liberan desde el axón presináptico a la hendidura sináptica y activan receptores en la dendrita postsináptica para transmitir la señal [3]. La formación de sinapsis y la posterior modificación de la estructura y la fuerza de la sinapsis están controladas por diversos factores, incluidos los factores secretados por los astrocitos vecinos (sinapsis tripartitas) [4].

El desarrollo cerebral comienza con el proceso de generación, diferenciación y migración de células neurales, luego continúa con el crecimiento axonal neuronal, formación de sinapsis y finalmente aparece el sistema de poda para esculpir los circuitos neuronales [5,6]. Siendo estas estructuras aún más perfeccionadas bajo el proceso de plasticidad sináptica. Por lo tanto, la plasticidad sináptica se refiere a la experiencia de cambios estructurales y funcionales mediados por las conexiones entre las neuronas que producen cambios en los circuitos neuronales [1].

Para entender bien el proceso de la memoria y la participación activa de la glía, es importante definir el concepto de plasticidad sináptica, el cual consiste en una modificación de la fuerza sináptica y una reorganización sináptica que se considera la base del aprendizaje y la memoria. La modificación de la fuerza sináptica se denomina plasticidad funcional y puede ir acompañada de un cambio en el número de sinapsis (plasticidad morfológica) [7].

Los procesos celulares de la plasticidad sináptica son críticos para la función cognitiva, el aprendizaje, la memoria, el comportamiento, la atención y la memoria de trabajo [8]. Ha sido sumamente discutido que las anomalías en la estructura de las sinapsis y la plasticidad funcional de las sinapsis, están implicadas en la fisiopatología de diversos trastornos psiquiátricos y neurológicos [5,9,10,11].

La plasticidad sináptica ha sido ampliamente estudiada en el hipocampo y los investigadores hablan de varios modelos: potenciación a corto plazo, potenciación a largo plazo (LTP) y depresión a largo plazo (LTD), todas las cuales se han asociado con la memoria [8,12]. Se ha demostrado que la plasticidad sináptica se ha mejorado en células recientemente generadas en el hipocampo [13], y también que estas nuevas células tienen una mayor LTP [14].

La plasticidad sináptica como hemos visto es compleja, pero a la vez, esencial para la ejecución de procesos neurocognitivos superiores. Donde la indemnidad de los complejos celulares es primordial.

En resumen, la potenciación a largo plazo es el fortalecimiento de larga duración de una conexión sináptica, mientras que la depresión a largo plazo debilita las sinapsis.

Considerando la activa y vital participación de los astrocitos en el control y fortalecimiento de las sinapsis, es necesario comprender la necesidad de la indemnidad de las células astrocíticas, en su estructura morfológica y en sus funciones homeostáticas particulares.

Uno de los factores moleculares necesarios para una neuroplasticidad adecuada es el factor neurotrófico derivado del cerebro (BDNF), que promueve la supervivencia de las neuronas existentes y estimula el crecimiento y la diferenciación de nuevas neuronas y sinapsis [15].

Muchos trastornos neuropsiquiátricos se caracterizan por una alteración dendrítica y sináptica, que incluye densidad y morfología de las espinas dendríticas anormales, pérdida de sinapsis y señalización sináptica y plasticidad aberrante.

Las patologías de la espina dendrítica o defectos en el desarrollo neuronal, incluyendo cambios en los patrones de ramificación, fragmentación, retracción o pérdida de ramificación de las dendritas y cambios en la morfología y número de las espinas dendríticas contribuyen a varios trastornos neurológicos y del

neurodesarrollo, tales como los trastornos del espectro autista, enfermedad de Alzheimer (AD), esquizofrenia, síndrome de Down (DS), ansiedad y depresión, entre otros [16,17].

Sobre la superficie dendrítica existen pequeñas protuberancias denominadas "espinas", también denominadas columnas dendríticas, (que comúnmente se categorizan de acuerdo a subtipos morfológicos, considerando el tamaño y proporción relativa de la cabeza y cuello de la espina) que es el sitio principal de la mayoría de las sinapsis excitatorias corticales y áreas principales para la plasticidad dinámica de la transmisión excitadora [18]. Estas espinas aumentan, en gran medida, el área de superficie disponible para la transmisión sináptica, lo que permite una alta densidad de conexiones sinápticas en dendritas y neuronas individuales. Las cabezas de las espinas contienen componentes postsinápticos y están conectadas a la dendrita mediante una estructura similar a un cuello [19].

Los 3 tipos más comúnmente categorizados son espinas “stubby” (pequeñas y aplanadas), “thin” (finas) y “mushroom” (en forma de hongos o setas) [19,20].

a) Las espinas pequeñas y aplanadas carecen de una distinción clara entre la cabeza y el cuello y se consideran un tipo inmaduro, son inestables y forman sinapsis comparativamente débiles [21].

b) Las espinas delgadas, tienen un cuello angosto y una cabeza relativamente pequeña, prevalecen durante el desarrollo y tienen una alta tasa de recambio.

c) Las espinas en forma de "hongo" tienen una cabeza grande, son las más maduras y estables, y el volumen de la espina se correlaciona positivamente con la fuerza de la sinapsis.

Asociado a lo anterior, existe otro tipo morfológico de estructura dendrítica, los llamados filopodios, que son espinas largas y delgadas que carecen de una cabeza distintiva [22]. Se cree que proporcionan un sustrato para el crecimiento y el fortalecimiento de las espinas dendríticas [23].

Las espinas más grandes en forma de hongo son relativamente estables y contienen sinapsis funcionales, mientras que las espinas delgadas, tipo filopodia, son muy lábiles, inmaduras e inestables [24].

Las espinas se forman y eliminan rápidamente en el cerebro en desarrollo y son más estables en el cerebro adulto [25]. Se ha propuesto que la pérdida de estabilidad de la sinapsis y las estructuras espinales, en las etapas posteriores de la vida, puede contribuir a los trastornos neurodegenerativos donde la memoria, el aprendizaje y la cognición están comprometidos [26].

En el trabajo ejecutado por Sultán et al, 2015, se destaca que los astrocitos desempeñan una función primordial y muy activa en los pasos cruciales del desarrollo de nuevas neuronas, más allá de la etapa de células madre o progenitoras [27].

2. Neurogénesis

Con base en la evidencia actualizada, la neurogénesis en el cerebro adulto está principalmente restringida a dos zonas principales:

a) La zona subgranular (SGZ) del giro dentado (GD) del hipocampo.

b) La zona subventricular (SVZ) que recubre los ventrículos laterales.

En el hipocampo, las células madre neurales multifuncionales, indiferenciadas, generadas en la GSZ dan lugar a células progenitoras neurales que proliferan y migran a la capa de células granulares del giro dentado y se diferencian en neuronas, astrocitos u oligodendrocitos.

Actualmente se estima que se generan aproximadamente 700 neuronas nuevas en el hipocampo de los seres humanos todos los días [14], y se acepta ampliamente que la neurogénesis en el adulto ocurre en el DG del hipocampo. La neurogénesis adulta es la forma más robusta de plasticidad en el cerebro adulto y probablemente contribuye a la formación de la memoria [28].

La neurogénesis adulta se puede dividir en tres eventos celulares principales: proliferación celular, diferenciación neuronal y supervivencia celular [29,30].

1. La proliferación celular se refiere a la división de las células progenitoras ubicadas en la SGZ de la circunvolución dentada. Tienen características morfológicas de la glía radial y expresan la proteína GFAP.
2. La diferenciación neuronal se refiere a la selección y aparición de un destino neuronal por parte de algunas células hijas. En la circunvolución dentada, la mayoría de las nuevas células se diferencian en neuronas. En los roedores, el porcentaje varía dependiendo de distintos factores, alcanzando entre 70-85% y un 10% aproximadamente se diferencian en glia [31].
3. La supervivencia celular se refiere al mantenimiento de nuevas neuronas y su incorporación permanente en el circuito del hipocampo.

En definitiva y en base a todo lo expuesto anteriormente, es crucial continuar con los esfuerzos en investigar exhaustivamente la estructura, función y mecanismos bioquímicos que encierran los astrocitos en su participación, cada vez más

sobresaliente, en los procesos de neurogénesis, plasticidad sináptica y sinaptogénesis, homeostasis neurocognitiva, neuroinflamación y cómo se ven afectados en condiciones extremas, tales como trauma, estrés, restricción de la movilidad en enfermedades neurodegenerativas.

C. NEUROGLIA

Como bien se señaló, la patología cerebral, hasta hace poco tiempo, estuvo sólo centrada en la neurona; en su supervivencia o su muerte. Esta visión puramente "neuronal" ahora está siendo reevaluada y la neuroglia comienza a ser considerada como un elemento central en la neuropatología [32,33,34].

Tanto las neuronas como la glía se originan en las células precursoras neuronales (células gliales radiales). Durante el desarrollo, las neuronas aparecen primero, seguidas por la glía, con numerosos factores extrínsecos e intrínsecos que participan en la transición de la generación celular [35].

El tejido neuroglial representa más del 50% de las células en el cerebro y se puede dividir en varios subtipos, de los cuales los astrocitos son los más abundantes. Aunque la existencia de astrocitos se documentó por primera vez hace más de 100 años, se han realizado relativamente pocos estudios sobre su papel en trastornos y enfermedades neurológicas [36].

La glía fue descrita inicialmente por Virchow en 1858 como *un tejido conectivo que une a los elementos nerviosos* [37]. Ramón y Cajal fue quién planteó la pregunta sobre cuál era la real función del tejido neuroglial, plasmando sus estudios en sus publicaciones: "Algo sobre la significación fisiológica de la neuroglia" (1897) y "Contribución al conocimiento de la neuroglia del cerebro humano" (1913). Posteriormente, Golgi introdujo la teoría de la función glial, en la que las células gliales eran responsables del soporte metabólico y el intercambio de sustancias en el cerebro [38].

Las células gliales se agrupan en tres categorías celulares principales en el SNC:[7]

1) Astrocitos, consideradas durante mucho tiempo sólo como células metabólicas de soporte de las neuronas.
2) Oligodendrocitos, presentes en la sustancia blanca, formando la vaina de mielina alrededor de los axones.
3) Microglia, identificadas como las células inmunes del cerebro.

Las células gliales desempeñan un papel activo en numerosos procesos fisiológicos que incluyen neurogénesis, sinaptogénesis [39] y plasticidad sináptica; transportan nutrientes y factores metabólicos esenciales para la supervivencia neuronal y funcionan desde la periferia hasta el cerebro y participan en la transmisión sináptica [7,40,41].

D. ASTROCITOS

Los astrocitos o astroglía son las células neurogliales más abundantes en el sistema nervioso central, participando activamente en numerosas funciones que permiten mantener la homeostasis neurocognitiva, incluyendo: la regulación del sistema de neurotransmisores y el procesamiento de la información sináptica, el metabolismo energético, la homeostasis iónica, el desarrollo y mantenimiento de la barrera hematoencefálica, las defensas antioxidantes y la respuesta inflamatoria [42].

Son células muy lábiles y se ven muy afectadas en condiciones de neurotoxicidad, neuroinflamación, trauma y estrés; aunque son mucho más resistentes a estos factores que el tejido neuronal. Son células multifuncionales, involucradas en la comunicación bidireccional con neuronas; al hacerlo, modulan la transmisión sináptica y la plasticidad [37,43].

Los astrocitos tienen un rol fundamental en la funcionalidad del cerebro, y su capacidad neuroprotectora ha sido motivo de variados estudios, como factor preventivo o terapéutico de muchas enfermedades neurodegenerativas [44]. Es importante destacar que cambios en la funcionalidad glial produce alteraciones en la respuesta inflamatoria, neurotransmisión y estrés oxidativo [45,46].

A los astrocitos, inicialmente, sólo se les confería una función pasiva funcional y estructural, sin embargo, hoy es ampliamente reconocida su participación activa en la integración, el procesamiento de la información [18] y la formación de memoria en el cerebro. La protección neuronal, el mantenimiento, el metabolismo, el soporte anatómico, la sinaptogénesis, la conectividad y transmisión sináptica, la homeostasis de electrolitos y glucosa y la producción de citoquinas son algunas de las funciones conocidas de estas células [40].

Los astrocitos desempeñan papeles importantes en la función neuronal. Coordinan la formación y la función de las sinapsis durante el desarrollo y proporcionan soporte trófico de referencia a las neuronas al acumular reservas de energía intracelular en forma de gránulos de glucógeno que pueden convertirse en lactato para suministrar a las neuronas [47].

La expresión de los receptores de insulina en los astrocitos demostró ser importante para la absorción de insulina y glucosa en el cerebro. En el hipotálamo, la falta de señalización de la insulina en los astrocitos afecta la respuesta normal a los cambios en la glucemia [48]. La memoria de discriminación requiere glucogenólisis, que se sabe que ocurre en astrocitos y no en neuronas [49], lo que sugiere que los subproductos del metabolismo del glucógeno son críticos para la formación de la memoria [50].

En contraste con la microglia cuyo origen es mieloide (médula ósea), los astrocitos (y los oligodendrocitos) se derivan de las células madre neurales que pertenecen embrionariamente a la placa ectodérmica (tubo y cresta neural) [22,51].

Según lo señalado por Allen, 2017, el nacimiento y desarrollo de los astrocitos generalmente ocurre después de que los períodos principales de neurogénesis y migración neuronal se completan en el cerebro de los mamíferos. Una excepción a esto se produce en el giro dentado del hipocampo y la región subventricular de la corteza cerebral, en la que se cree que los astrocitos residentes proporcionan señales que participan en la generación de nuevas neuronas [52].

Los astrocitos comparten algunas propiedades con células madre neurales y crean un entorno propicio para la neurogénesis, siendo participantes activos en el nicho neurogénico. Regulan la neurogénesis mediante la secreción de factores tales como Wnt3 [53], interleuquina-1β, interleuquina-6 y proteína 6 de unión al factor de crecimiento similar a la insulina (IGFBP6) [54].

Sin duda, los astrocitos son más que unas simples células que apoyan metabólicamente a las neuronas. He aquí una síntesis de algunas funciones [18]:

- La homeostasis de iones, agua y neurotransmisores [55].
- Neurogénesis, gliogénesis y crecimiento de neuritas [56].
- Desarrollo de sinapsis, función y plasticidad [57].
- Detección y transporte de glucosa; almacenamiento de energía [58].
- Regulación del flujo sanguíneo [59].
- Procesamiento sensorial, coordinación motora, emoción y cognición [60].

Avalados en la gran cantidad de investigaciones, realizadas en los últimos 20 años, se ha descrito que los astrocitos ubicados en la materia gris (astrocitos protoplásmicos) son componentes integrales de las sinapsis, participando activamente en el control de la formación y función de sinapsis. Estos astrocitos poseen procesos extensamente ramificados que terminan en estructuras finas, denominados procesos astrocíticos perisinápticos (PAP), que estructural y funcionalmente interactúan con las sinapsis [61].

Las diversas investigaciones sobre la relación astrocito-sinapsis-neurona han revelado que los astrocitos forman un componente integral de la arquitectura del sistema nervioso, siendo reguladores críticos de la función cerebral y la plasticidad, a través de interacciones dinámicas y bidireccionales con las sinapsis [62]. Estas observaciones indican que los astrocitos, a través de sus procesos finos, tienen la capacidad de detectar y adherirse a las sinapsis y coordinarse con los astrocitos vecinos para recubrir y cubrir completamente el neuropilo [63].

Los astrocitos encierran muchos contactos sinápticos y, por lo tanto, ayudan a asegurar la excitabilidad neuronal normal manteniendo la homeostasis iónica extracelular a través del aclaramiento de iones de potasio y glutamato de las regiones alrededor de las sinapsis. Los astrocitos, al sintetizar glutamina, que utilizan las neuronas para formar glutamato, también contribuyen significativamente a la homeostasis metabólica neuronal [64].

Considerando las conclusiones de varios estudios de microscopía electrónica, al parecer, sólo una fracción de las sinapsis está en contacto inmediato con los procesos astrocíticos, en un momento determinado. Se estima que, en roedores, un 30 a 60% de las sinapsis están envueltas por astrocitos en la neocorteza, mientras que un 60 a 90% de las espinas están en contacto con un proceso astrocítico en el hipocampo y un 90% en la capa de la corteza somatosensorial IV [65].

Los astrocitos humanos son más grandes, más complejos y ramificados que, por ejemplo, los astrocitos de roedores [66], cobrando mucha importancia para las capacidades únicas de procesamiento del cerebro humano. Desde el soma celular irradian ramas primarias que gradualmente se dividen en procesos cada vez más finos (abarcan casi 1 mm) [67].

Los astrocitos humanos, como los astrocitos de roedores, están organizados en dominios. Dependiendo de la región cerebral en particular, un solo astrocito maduro de roedor puede cubrir un dominio espacial en el cerebro que oscila entre 20,000 y 80,000 μm3, envolver múltiples somas neuronales, asociarse con 300 a 600 dendritas neuronales, y tomar contacto con ~ 100,000 sinapsis individuales. En los humanos, estos números aumentan dramáticamente, con un solo astrocito ocupando un volumen en el cerebro que es casi 30 veces el volumen en roedores y se asocia con aproximadamente 2 millones de sinapsis [68].

Los astrocitos influyen en múltiples aspectos de la transmisión sináptica al mantener la homeostasis extracelular. Como resultado, son fundamentales para promover la supervivencia neuronal en el contexto de la neuroinflamación y la hipoxia, entre otros [69,70]. Son también participantes activos en la plasticidad estructural, durante el establecimiento inicial y la remodelación continua de las conexiones sinápticas dentro del sistema nervioso. Los astrocitos también liberan varios factores solubles para controlar la maduración de las sinapsis [71].

Diversos estudios han destacado que los astrocitos reaccionan a la estimulación neuronal cambiando su morfología y se observa, en sus proyecciones específicas de la región estimulada, un aumento significativo en la envoltura astrocítica de sinapsis excitatorias en las espinas dendríticas [72].

El cerebro consume aproximadamente el 20% total de ATP producido, siendo utilizado principalmente para la repolarización de la membrana y el transporte intracelular. Las células gliales sirven como importantes centros de transferencia al tomar la glucosa (principal fuente energética) y distribuir el lactato (derivado de la glucólisis), a través de la lanzadera de lactato, a compartimentos neuronales distantes, como las sinapsis y los axones mielinizados [73].

Los astrocitos mantienen una gran tasa glucolítica y muestran una alta expresión de enzimas muy importantes para este proceso (por ejemplo, 6-fosfofructosa-2-quinasa, fructosa-2,6-bifosfatasa-3) [74]. Participan en la regulación de la entrada de nutrientes en el cerebro, actuando como sensores metabólicos y promoviendo la supervivencia neuronal y el mantenimiento de la homeostasis del SNC [40].

Suzuki et al, 2011, proponen que el transporte de lactato de astrocitos y neuronas es esencial para la plasticidad sináptica a largo plazo, la memoria a largo plazo y sus cambios moleculares y sinápticos subyacentes. Estos resultados pueden tener implicaciones importantes para los trastornos de la memoria y las patologías que conllevan déficits cognitivos y de memoria en general, que incluyen afecciones neurodegenerativas como la enfermedad de Alzheimer, el envejecimiento y la demencia [50].

Una de las principales funciones de los astrocitos es tomar el glutamato extracelular, un proceso que se produce a través del transportador de glutamato 1 (EAAT2/GLT-1) y el transportador de glutamato/aspartato (EAAT1/GLAST), ambos de los cuales están altamente enriquecidos en los procesos astrocíticos [75,76].

Los astrocitos son responsables de eliminar el glutamato extracelular, ya que posee transportadores de glutamato con una alta afinidad (GLAST y GLT-1). Para concluir este proceso el astrocito convierte el glutamato en glutamina mediante la glutamina sintetasa (GS), que es expresada absoluta y abundantemente en astrocitos. La GS es muy sensible al estado oxidativo y nitrosativo, lo que afecta directamente la neurotransmisión excitatoria e inhibitoria, que aumenta en estados de estrés, restricción de movimiento y envejecimiento [42].

Diversos estudios experimentales, in vitro, muestran diferencias en la expresión de los transportadores durante el desarrollo, GLAST predominantemente en etapas tempranas y GLT-1 en la madurez.

Aunque los astrocitos no se han clasificado tradicionalmente en función de sus propiedades fisiológicas, varios estudios han descrito astrocitos electrofisiológicamente "complejos" y "pasivos". Los astrocitos también difieren en su expresión de uniones gap, el grado de acoplamiento, y la conectividad de las redes astrocíticas es altamente específica [77].

Los astrocitos se clasifican en función de su morfología, según el tamaño del cuerpo celular, el número de procesos, el grosor de los procesos, la dirección de los procesos o la longitud de los procesos en tipos I, II y III. Los astrocitos tipo I tienen un tamaño de cuerpo celular pequeño y numerosos procesos cortos, los astrocitos tipo II tienen una forma bipolar y procesos largos. Los astrocitos de tipo III se caracterizan por una forma de estrella y procesos largos [78,79].

Los resultados de Guerreiro et al, 2016, mostraron que los ratones que viven toda la vida en un entorno empobrecido pierden diversidad morfológica astrocítica. Por el contrario, los individuos mantenidos al mismo tiempo en un ambiente enriquecido no perdieron diversidad astrocítica. De hecho, 6 meses de ambiente enriquecido aumentan 113% el número de astrocitos de mayor complejidad, y el número absoluto de estos astrocitos tipo I no se reduce más adelante en la vida [72].

Se reconocen al menos tres subtipos morfológicos de astrocitos en el cerebro, los astrocitos protoplásmicos, fibrosos y radiales [22].

a) **Astrocitos protoplásmicos de la materia gris** (más abundantes de la corteza humana, residen en las capas II-VI) [78], exhiben una morfología muy ramificada, sus procesos envuelven en un extremo a las sinapsis neuronales, mediante sus procesos perisinápticos (extremadamente finos) para infiltrarse en el neuropilo circundante (originados de sus ramas secundarias y terciarias) y en el otro extremo encierran vasos sanguíneos. Las zonas que rodean la región sináptica están densamente enriquecidas con transportadores de glutamato y tienen un papel principal en el aclaramiento de glutamato en condiciones fisiológicas [80].
   Los astrocitos protoplásmicos humanos tienen un diámetro 2,55 veces mayor y tienen procesos que son 2,6 veces más largos, 1,3 veces más gruesos y 10 veces más inmunorreactivos GFAP, que los astrocitos protoplásmicos de roedores [77].

b) **Astrocitos fibrosos de la materia blanca**, se asocian con el tracto axonal mielinizado, el cuerpo calloso y toman contacto con los nodos de Ranvier. Actualmente se consideran una población muy diversa, con funciones específicas e incluso diferentes, dependiendo del lugar en el SNC donde estén localizados o el período de desarrollo en el cual se encuentren [62]. Estos procesos astrocíticos fibrosos protegen y nutren las regiones no mielínicas, en oposición a los oligodendrocitos que protegen y nutren la vaina de mielina [80]. Los astrocitos fibrosos (además de desempeñar las mismas funciones que los astrocitos protoplasmáticos), también tienen un papel destacado en la reparación de las neuronas dañadas y conducen a la formación de cicatrices [22].

c) **Astrocito "radial"**, se desarrollan a partir de células madre neurales durante la embriogénesis temprana para ayudar en la migración neuronal y en el desarrollo de la placa neural. Se sabe que el astrocito radial persiste en la retina y el cerebelo adulto como células de Müller y Berger respectivamente [81].

Se ha informado que el volumen de neuropilo que ocupa un astrocito en el cerebro de roedores varía de 14.700 a 22.906 μm^3 en la corteza y de 65.900 a 85.300 μm^3 en el hipocampo. Se ha encontrado hasta ahora que el tamaño de los astrocitos protoplasmáticos en diferentes especies es proporcional al aumento en la complejidad de las funciones cerebrales superiores [43,77].

Los astrocitos tienen el potencial de cambiar su morfología, hipertrofia y atrofia, en reacción a cualquier condición patológica, en su interacción con neuronas, así como con otras células no neuronales. Estos cambios morfológicos ocurren en su cuerpo celular y procesos proximales que rodean a las sinapsis y envuelven los nódulos axonales. Se han informado cambios morfológicos en astrocitos en lesiones cerebrales traumáticas, accidentes cerebrovasculares, isquemia y enfermedades neurodegenerativas. La hipertrofia de astrocitos ha sido reportada durante condiciones neurodegenerativas como AD y PD, mientras que los informes de atrofia de astrocitos han sugerido principalmente su correlación con la función inmune, con el envejecimiento normal y el estrés crónico [51,82,83,84].

La expresión de GFAP se ha convertido en el marcador prototípico para la identificación inmunohistoquímica de los astrocitos. Su patrón de expresión difiere en las distintas regiones del sistema nervioso central. En la corteza y el hipocampo, el marcaje con GFAP de astrocitos protoplasmáticos revela la apariencia típica en forma de estrella de los astrocitos y, en el cerebelo, una apariencia radial [77].

Los astrocitos se caracterizan por tener una morfología estrellada, que cambia a un estado reactivo bajo condiciones de estrés y degenerativas. Esta morfología individual está directamente relacionada con la expresión de GFAP. La regulación al alza de GFAP depende de la naturaleza del daño, la distancia entre el astrocito y el sitio de la lesión, y el tiempo después de la lesión [85].

En el cerebro adulto, los pies astrocíticos forman una red de recubrimiento alrededor de la vasculatura del cerebro conocida como glia limitans, que junto con los pericitos y las células endoteliales forman una barrera para el paso de moléculas, iones y células del torrente sanguíneo al parénquima cerebral, la barrera hematoencefálica (BHE).

Se conoce también que los astrocitos desempeñan un papel muy importante en la regulación del flujo sanguíneo cerebral y en la regulación de la permeabilidad de la BHE del torrente sanguíneo al parénquima cerebral. Los defectos en la BHE están

implicados en muchos estados de enfermedad en adultos, incluidas enfermedades neurodegenerativas y neuroinflamatorias [69].

Los astrocitos no son eléctricamente excitables, pero exhiben señales de calcio intracelulares, inducidas tanto por estímulos como espontáneas (aparentemente en ausencia de estimulación o actividad neuronal). El calcio es un segundo mensajero ubicuo para muchas vías de señalización, los aumentos transitorios en el calcio intracelular pueden activar muchos factores de transcripción y enzimas aguas abajo como PKC, NFkB, CaMKII y CREB [65]. Los astrocitos del tejido cerebral humano muestran "excitabilidad intrínseca" basada en Ca^{2+} y pueden responder a neurotransmisores liberados sinápticamente [86]. Los astrocitos humanos exhiben una alta expresión de proteínas implicadas en la señalización de Ca^{2+} y propagan las ondas de Ca^{2+} a velocidades mucho más rápidas que sus contrapartes de roedores [87].

Empleando una variedad de mecanismos moleculares (exocitosis, transportadores de membrana o difusión a través de canales plasmalémicos), los astrocitos secretan numerosos neurotransmisores, neurohormonas y factores tróficos que regulan la formación y mantenimiento de sinapsis, modulan la transmisión sináptica y la plasticidad después de la "activación", que está representada por cambios en señales de calcio citoplásmico en respuesta a la actividad neuronal o a la información sináptica [2].

Para mantener la concentración extracelular de los neurotransmisores, los astrocitos eliminan rápidamente el K + acumulado como resultado de la actividad neuronal, absorben el glutamato liberado durante la neurotransmisión y convierten el glutamato en glutamina y lo liberan nuevamente a las terminales presinápticas. El glutamato juega un papel clave en la regulación de la actividad sináptica y causa una respuesta en los astrocitos [52].

El término "sinapsis tripartita" se introdujo por primera vez hace más de dos décadas para describir esta relación íntima entre las neuronas y los astrocitos en las sinapsis glutamatérgicas y ahora se ha extendido a otras sinapsis, como la sinapsis tripartita monoaminérgica [18].

Recientemente se ha demostrado que la integración sináptica de las neuronas hipocampales del recién nacido está controlada localmente por los astrocitos. El bloqueo de la liberación vesicular de los astrocitos produce una menor densidad de la columna en las células recién nacidas, pero sólo en las dendritas neuronales [88,89].

Los astrocitos expresan numerosos receptores de neurotransmisores, canales iónicos y sistemas de segundo mensajero y, por lo tanto, pueden responder e integrar los aportes de las neuronas. Por lo tanto, los astrocitos no sólo participan en el control homeostático pasivo de condiciones adecuadas para la función

sináptica, sino que también modulan activamente la excitabilidad neuronal y la transmisión sináptica en la función sináptica, contribuyendo al rendimiento funcional del cerebro a través de la actividad coordinada de redes neuronales complejas que comprenden neuronas y glía [71].

La expresión astroglial de una amplia gama de receptores para neurotransmisores y neurohormonas está regulada por el entorno neuroquímico y, por regla general, los astrocitos poseen receptores que les permiten detectar la transmisión neuronal vecina. La activación de estos receptores desencadena cambios dinámicos de concentración de iones (principalmente Ca^{2+} y Na^{+}) en el citoplasma astroglial, que regulan las funciones astrogliales y sirven como sustrato para la excitabilidad astroglial [2,37,90].

Los astrocitos juegan papeles directos e interactivos con neuronas en la transmisión sináptica a través de la liberación regulada de moléculas sinápticamente activas que incluyen glutamato, purinas (ATP y adenosina), GABA y D-serina. El glutamato liberado de los astrocitos activa los receptores presinápticos de NMDA y promueve el aumento de la comunicación excitatoria entre las neuronas [43,71,91].

La señalización glial se produce por la transferencia directa de neurotransmisores (como glutamato) e iones (como Ca^{2+} y Na^{+}) de una célula glial a otra a través de uniones gap. Un gran número de astrocitos se conectan entre sí, a través de tales uniones gap, para formar sincitios astrogliales conectados, capaces de transmitir información de señalización transináptica, a través de grandes regiones del cerebro [22].

Los astrocitos están ampliamente acoplados a través de uniones gap, compuestas de proteínas conexina (CX) predominantemente de los subtipos CX-43 y CX-30, que permiten el movimiento intercelular de metabolitos e iones a grandes distancias. La alta densidad de los canales de agua de la acuaporina 4 (AQP4) y los transportadores de iones en los procesos de pie astrocítico son importantes en la homeostasis de los fluidos y edema vasogénico y citotóxico después de la isquemia, trauma e inflamación [74].

Además, debido a que un solo astrocito puede estar en contacto con miles de sinapsis, las elevaciones de calcio astrocítico y posterior liberación de glutamato conducen a la excitación sincrónica de grupos de neuronas, lo que indica que la gliotransmisión puede contribuir a la sincronización neuronal [52].

Otras funciones de astrocitos, importantes a destacar [92]:

- Regulación del flujo sanguíneo a través de la liberación de mediadores tales como ácido araquidónico, prostaglandinas y óxido nítrico.

- Contribución a la formación, mantenimiento y poda de las sinapsis durante el desarrollo.
- Capacidad de respuesta al estrés oxidativo produciendo glutatión, ácido ascórbico y superóxido dismutasas [93].

Un corolario de esto es la idea de que los astrocitos pueden participar en la plasticidad sináptica en regiones como el hipotálamo y el hipocampo. En el SNC adulto, también se sabe que los astrocitos cumplen varias funciones regionales. Por ejemplo, en la SVZ, una región germinal del cerebro adulto, las células astrocíticas que expresan GFAP sirven como células madre / progenitoras que dan lugar a neuronas adultas en el bulbo olfatorio de los roedores [69,86].

La plasticidad adaptativa implica arborización dendrítica y axonal, densidad de la espina, número y tamaño de sinapsis (plasticidad sináptica estructural), así como cambios en la composición y densidad del receptor y regulación de la liberación de neurotransmisores que involucra sinapsis individuales (plasticidad sináptica Hebbiana, Hebb 1949) o restablecer la fuerza de todas las sinapsis en una neurona particular (escala sináptica homeostática) [32].

Los astrocitos son participantes activos en la plasticidad estructural durante el establecimiento inicial y la remodelación continua de las conexiones sinápticas dentro del sistema nervioso. Los astrocitos también liberan varios factores solubles distintos para controlar la maduración de las sinapsis. Los glipicanos 4 y 6 derivados de astrocitos son necesarios y suficientes para promover la agrupación y receptividad del receptor de glutamato e inducir la formación de sinapsis que funcionan postsinápticamente [52].

Hoy es aceptado que los astrocitos se encuentran en dos estados, en reposo y en estado reactivo. Los cambios morfológicos en el estado reactivo incluyen incremento del espesor y número de procesos, aumento en el tamaño del cuerpo celular y expresión de la proteína GFAP. Sin embargo, los cambios morfológicos y moleculares durante el proceso de aprendizaje y memoria no han sido dilucidados. Recientes estudios se centran ampliamente en los papeles de los astrocitos en la formación sináptica y la neurogénesis, y en el apoyo a la formación de aprendizaje y la memoria [78].

Los factores tróficos liberados por los astrocitos (GDNF, BDNF, S100B y TGF-β) son fundamentales para la proliferación, supervivencia y maduración de células neuronales, cumpliendo un rol protector, reparador y como biomarcador. Se considera también a los astrocitos del hipocampo como promotores de la neurogénesis [42].

La secreción dependiente de actividad de BDNF potencia la plasticidad sináptica. El BDNF, que se sintetiza tanto en neuronas como en astrocitos, se secreta en su forma precursora (pro-BDNF) y luego se elimina del espacio extracelular mediante la absorción rápida por astrocitos cercanos [94], sugiriendo que los astrocitos ejercen una función importante en el aclaramiento neuronal del pro-BDNF secretado de la actividad neuronal y posterior reciclaje de la neurotrofina endocítica. El reciclaje de BDNF por los astrocitos puede contribuir a la regulación de la plasticidad sináptica por parte de la glía [52].

BDNF es un candidato plausible capaz de afectar procesos de memoria debido a su papel crítico en procesos de plasticidad sináptica de fase tardía y memoria a largo plazo. Se ha demostrado que el BDNF se induce de manera rápida y selectiva en el hipocampo luego del condicionamiento contextual del miedo [95].

A su vez, la reducción de BDNF inducida por neuroinflamación dio lugar a déficits de memoria dependientes del hipocampo [95,96]. El aprendizaje espacial y la memoria de trabajo en ratas parece aumentar el número de astrocitos [50].

Estudios recientes muestran que la inhibición de la apoptosis de astrocitos es una estrategia neuroprotectora esencial [97].

E. GFAP

La proteína ácida fibrilar glial es una proteína que forma los filamentos intermedios del citoesqueleto de las células astrogliales que provee soporte para la estructura y movimiento celular, comunicación celular y barrera hematoencefálica. Las proteínas intermedias de filamento, GFAP y vimentina, se expresan en astrocitos y parecen afectar críticamente los procesos mediante los cuales los astrocitos controlan la neurogénesis y otros aspectos de la plasticidad y regeneración neuronal. Los astrocitos expresan 10 isoformas diferentes de GFAP, junto con vimentina, nestina y sinemina [98].

Es el marcador clásico para la identificación inmunohistoquímica de astrocitos. Se aisló por primera vez en placas de pacientes con esclerosis múltiple, constituidas por axones desmielinizados y astrocitos fibrosos [99].

La expresión de GFAP se usa ampliamente para la identificación de astroglia in vivo e in vitro, y la regulación positiva de este marcador en astrocitos es un sello distintivo típico de las patologías del sistema nervioso central [100].

En cultivo celular el análisis inmunohistoquímico muestra un intenso marcaje citoplasmático para GFAP. Expresión de GFAP bastante menor cuando es comparado cultivos de astrocitos de adultos o envejecidos con cultivos celulares de recién nacidos [42]. La expresión de las proteínas del filamento intermedio del citoesqueleto (incluido GFAP), que se observa típicamente en los astrocitos, aumenta con la edad [75].

La formación de procesos astrocíticos estables, en respuesta a las neuronas, requiere la presencia de GFAP [101]. Se observa un decrecimiento importante de la expresión de GFAP que podría reflejar la degeneración astroglial, la cual ha sido detectada en los tempranos estadios de las enfermedades neurodegenerativas asociadas a la edad [42].

Los astrocitos emplean su red de filamentos intermedios que contiene GFAP como una plataforma de señalización y un andamio estructural que coordina las respuestas apropiadas de los astrocitos en la salud y la enfermedad [98]. GFAP es estructuralmente similar a otros miembros del filamento intermedio (IF) no epiteliales (clase III), que incluyen vimentina, desmina y periferina (que se expresa en neuronas periféricas y células ganglionares entéricas).

GFAP es responsable de la estructura del citoesqueleto de las células gliales y de mantener su resistencia mecánica, así como para apoyar a las neuronas vecinas y la barrera hematoencefálica [98].

La expresión de GFAP no es exclusiva de astrocitos protoplásmicos y fibrosos; también expresada por glía de Müller en retina, glía de Bergmann en el cerebelo, tanicitos en la base del tercer ventrículo o pituicitos en la neurohipófisis, entre otros.

Las células astrogliales responden a lesiones cerebrales y otras condiciones neuro-perturbadas al someterse a "astrogliosis reactiva", un proceso mediante el cual las células astrogliales experimentan hipertrofia celular (aumento de tamaño y expresión de GFAP) y proliferación (aumento del número de células gliales) [102].

Considerando que GFAP (junto con vimentina), es el responsable del ensamblaje y extensión del filamento intermedio, dentro de los procesos astrocíticos, se cree que la inducción de GFAP es muy importante para la formación de procesos astrocíticos extendidos y engrosados observados en gliosis reactiva.

Se ha comunicado también que el GFAP tiene participación activa en la migración celular y la motilidad, en el anclaje del transportador de glutamato GLAST / EAAT1 en la membrana y en la mitosis [103].

La sobreexpresión de GFAP resultó en una encefalopatía fatal con acumulaciones de GFAP en fibras de Rosenthal. Esta afirmación llevó al descubrimiento de que la enfermedad de Alexander es causada por mutaciones en el gen GFAP [104]. Un aumento en la proteína GFAP es una característica prominente en enfermedades degenerativas como PD y AD. Se encuentra que los astrocitos activados (con GFAP altamente expresado) rodean las placas de amiloide y neurita en AD [102].

F. HIPOCAMPO

Se ha establecido, desde hace ya bastante tiempo, que el hipocampo es vital para el aprendizaje y la memoria, pero estudios actuales sugieren que también desempeña un importante papel en la regulación del estado de ánimo [1,105].

El hipocampo es crítico para el aprendizaje contextual y espacial y la conciencia, la navegación y los recuerdos episódicos [95].

El hipocampo es un componente clave del sistema de memoria espacial y episódica, tanto en humanos como en roedores, y es particularmente vulnerable a los efectos del estrés severo y prolongado. La acumulación de evidencia de neuroimágenes y estudios clínicos, sobre trastornos psiquiátricos, relacionados con el estrés han reportado una reducción en el volumen del hipocampo, y se cree que esto contribuye a los déficits de memoria observados en estas condiciones debilitantes [106].

El hipocampo es un área clave del cerebro involucrada en la regulación de la respuesta al estrés, que ejerce una retroalimentación negativa sobre el eje hipotalámico pituitario adrenal (HPA) [107].

Estudios en los cuales se anuló la neurogénesis en el hipocampo de los roedores han demostrado una disminución del rendimiento cognitivo en tareas dependientes de la memoria del hipocampo como el laberinto acuático de Morris, el miedo contextual, reconocimiento espacial y de objetos [108].

La neurogénesis adulta parece ser funcional dado que su abolición afecta el aprendizaje dependiente del hipocampo [109].

Estructuralmente el hipocampo posee diversas zonas funcionales que participan en la formación de la memoria, CA1, CA3 y la circunvolución dentada o giro dentado (DG). Varias partes del cerebro muestran alguna forma de plasticidad sináptica, pero el hipocampo es una de las estructuras que ha recibido mucha atención debido a su importancia funcional general [1].

Las neuronas excitadoras son principalmente neuronas piramidales y constituyen el 90% de todas las neuronas en el hipocampo, mientras que el 10% restante son interneuronas, principalmente inhibidoras, que se clasifican según sus características morfológicas, fisiológicas, moleculares y sinápticas en otras numerosas subclases [110].

Hasta ahora, las investigaciones moleculares y celulares de la formación y el almacenamiento de la memoria se han centrado principalmente en los mecanismos neuronales. Sin embargo, el trabajo experimental reciente ha comenzado a hacer

preguntas sobre los roles de las células gliales, en el procesamiento de información codificada y el almacenamiento de recuerdos [111].

El daño al hipocampo, y en particular los efectos del daño sobre los mecanismos de plasticidad sináptica que soportan la memoria, tendrán, por lo tanto, profundas implicaciones para la cognición [112].

El estrés crónico y los glucocorticoides afectan la función del hipocampo, lo que a su vez contribuye a la desregulación del eje de la HPA [113].

Además de su papel en la regulación del eje HPA, el hipocampo es una estructura bastante única ya que es una de las pocas áreas en el cerebro de mamífero sano donde la neurogénesis, el nacimiento de nuevas neuronas, ocurre a lo largo de la vida adulta. La neurogénesis en el hipocampo (adulto) se produce en la zona subgranular y se compone de varias etapas: proliferación celular, diferenciación y supervivencia neuronal y maduración de las neuronas recién nacidas [29,107].

Los estudios en el hipocampo han estado a la vanguardia de la investigación de plasticidad sináptica desde los descubrimientos originales de LTP y LTD, de la transmisión sináptica en esta región del cerebro [112]. Algunos estudios han demostrado que la estimulación directa de los astrocitos individuales en el hipocampo y en el hipotálamo, provocan una potenciación duradera de las sinapsis [7].

El hipocampo posee receptores mineralocorticoides (MR, tipo I) y receptores glucocorticoides (GR, tipo II), los MR median los efectos de los glucocorticoides sobre la evaluación del factor estresante y el inicio de la respuesta al estrés, mientras que los GR actúan en la consolidación de la información adquirida [107].

El hipocampo de roedores contiene altos niveles de receptores de glucocorticoides (GR) y receptores de tipo mineralocorticoides (MR). La afinidad de MR por corticosterona es de 6 a 10 veces mayor que la de GR, pero es GR que se activa después del estrés y participa en su acción de retroalimentación sobre la plasticidad neuronal inducida por el estrés. El estrés crónico disminuye la expresión de GR y finalmente altera el equilibrio de GR / MR en el hipocampo masculino; se piensa que esto es un mecanismo de protección contra los efectos dañinos del estrés crónico [113, 114].

El DG es un área del cerebro caracterizada por una población grande y densa de células granulares glutamatérgicas con una actividad muy escasa. Es una región de entrada importante para el hipocampo y, por lo tanto, se cree que desempeña un papel esencial en el aprendizaje, la memoria episódica y las tareas de navegación espacial asociadas con esa estructura [28].

Las reducciones en la proliferación celular, la supervivencia [115] y la diferenciación neuronal [116], cambios en la estructura y complejidad neuronal [117], así como también deterioro en tareas de memoria asociadas al hipocampo han sido observados en modelos de AD [108, 118].

Los estudios de resonancia magnética (RM) indican que el volumen del hipocampo está disminuido en pacientes con depresión mayor -uno de los únicos cambios físicos detectables asociados con esta enfermedad- y los pacientes con más episodios depresivos muestran una mayor pérdida de volumen del hipocampo. Después del tratamiento antidepresivo el volumen del hipocampo se puede normalizar, lo que se correlaciona con una disminución de los síntomas [105].

1. Memoria

Se cree que la plasticidad sináptica del hipocampo, modelada por la LTP, representa un mecanismo importante de la formación de memoria dependiente del hipocampo [119]. Se ha aceptado ampliamente que los recuerdos se almacenan, al menos en parte, como cambios en la fuerza de las conexiones sinápticas entre las neuronas y astrocitos, es decir, a través de la plasticidad sináptica.

Varios estudios han demostrado la participación activa de los astrocitos en la formación de la memoria [64] y su relación con la LTP, importante para la plasticidad sináptica, el aprendizaje y la memoria [98].

La metaplasticidad permite o inhibe la plasticidad sináptica a lo largo del tiempo, a través de la regulación de la actividad celular, potenciación o debilitamiento de la actividad sináptica, dentro de un rango dinámico, mediante la modificación de los umbrales de LTP y LTD [64]. La metaplasticidad es, por lo tanto, crítica para la función y salud neuronal [112].

Los astrocitos no sólo regulan la concentración extracelular de neurotransmisores, también regulan la actividad y expresión de los receptores en la neurona postsináptica, a través de la actividad de los gliotransmisores, y desempeñan un importante papel en la amortiguación de la actividad y en la eliminación de conexiones no ventajosas [1].

Choi et al. (2016) investigaron los cambios morfológicos y moleculares en astrocitos, basados en la hipótesis de que estos cambios estaban acompañados por la inducción de la memoria a largo plazo en el giro dentado del hipocampo. Sus resultados mostraron que la memoria contextual, con base en el hipocampo, indujo un aumento de las prolongaciones de astrocitos tipo II y tipo III, pero se observaron niveles disminuidos de proteína GFAP. Concluyendo que es necesario un estado astrocítico alterado para inducir el proceso de aprendizaje y memoria [78].

Los astrocitos ejercen una variedad de influencias en los procesos neurales críticos para la memoria, como regulación de la concentración extracelular de K^+ y Ca^{2+} intracelular, soporte metabólico a las neuronas (glucosa o lactato), reciclamiento de glutamato/GABA, liberación de gliotransmisores para la regulación neuronal, regulación del flujo sanguíneo.

Suzuki et al., 2011, divulgaron que, en el hipocampo de la rata, el aprendizaje conduce a un aumento significativo en los niveles de lactato extracelular que se derivan de glucógeno, una reserva de energía selectivamente localizada en los astrocitos. La descomposición del glucógeno astrocítico y la liberación de lactato son esenciales para la formación de memoria a largo plazo, pero no a corto plazo. La glucogenólisis y los transportadores de lactato astrocítico también son críticos para la inducción de los cambios moleculares necesarios para la formación de la memoria, incluida la inducción de fosfo-CREB, Arc y fosfo-cofilina [50].

Como se discutió anteriormente, otro mecanismo por el cual los astrocitos pueden contribuir al aprendizaje y la memoria es a través del metabolismo de la glucosa que conduce a la exportación de lactato, que a la vez regulará el flujo sanguíneo cerebral y potencialmente proporcionará soporte metabólico a las neuronas activas.

2. Disfunción y Reactividad Astrocítica

Los astrocitos cumplen una función fundamental en la homeostasis cerebral y, considerando la fuerte relación metabólica entre las neuronas y los astrocitos, se puede postular que la disfunción astrocítica puede conducir a muchas enfermedades neurológicas. Estas enfermedades neurológicas comparten procesos fisiopatológicos comunes, como el estrés oxidativo, excitotoxicidad, falla metabólica o inflamación, muchos de los cuales son contrarrestados por astrocitos en el cerebro sano [120].

Los astrocitos en el tejido sano tienen microdominios no superpuestos, claramente definidos, que crean una arquitectura compleja para modular el disparo neuronal sináptico con el flujo sanguíneo y para controlar la actividad neuronal [43,67,121].

Cuando se produce una injuria en el sistema nervioso central, los astrocitos detectan los cambios moleculares en su entorno extracelular y en las células a las que están acoplados, y alteran sus características morfológicas y funcionales para adoptar un fenotipo reactivo. Como es de esperar, por sus ubicaciones en todo el cerebro y sus funciones en el cerebro normal, los astrocitos se vuelven reactivos cuando captan la lesión [47].

La fuerte expresión de GFAP se convirtió en el sello distintivo de los astrocitos reactivos en la década de 1970. Además de la regulación al alza de GFAP, los astrocitos reactivos alteran su morfología y expresión génica.

Debido a estas funciones diversas y críticas en el sistema nervioso central (SNC) sano y disfuncional, es primordial apreciar cómo las adaptaciones o la disfunción de los astrocitos contribuyen a la etiología de la enfermedad neuropsiquiátrica [121].

Durante la polarización, los astrocitos alargados se agregan en haces agrupados primero ortogonales, y luego paralelos al sitio de la lesión. Estos haces de astrocitos y las células inmunes invasoras rodean el tejido lesionado. Aunque los astrocitos más alejados del sitio de la lesión también se hipertrofian, no forman haces y conservan sus dominios originales que no se superponen [47].

La astrogliopatía es un elemento clave en la patogénesis y la patología de varias enfermedades neurológicas, incluidas las enfermedades degenerativas del sistema nervioso central [92].

Es así como, Pekny et al., 2016, han propuesto dos vías de astrogliopatía: la astrogliosis o astrocitosis reactiva, comúnmente conocida, donde los astrocitos pueden tener efectos neuroprotectores (formación de cicatriz glial), y una segunda vía llamada astrocitopatía, que incluye atrofia/degeneración, con pérdida de función y remodelación patológica de astrocitos [32, 121].

Los cambios dinámicos de los astrocitos no se limitan sólo a la astrogliosis, sino también se han descrito astrocitos atróficos, donde los astrocitos son más pequeños y menos arborizados. La astrogliopatía abarca el concepto fundamental de la gliodegeneración [122] y destaca el papel cardinal de la disfunción astrocítica en la patogénesis de las enfermedades neurológicas [34].

Con base en lo planteado anteriormente, la astroglía representa el elemento homeostático y regulador fundamental del SNC; sin embargo, su disfunción contribuye a la patogénesis de la mayoría de las enfermedades neurológicas, a través, de vías múltiples y complejas cuya respuesta va desde la astrogliosis reactiva a la astrodegeneración y/o la remodelación patológica, con pérdida o modificación de la función [32].

3. Astrogliosis o Astrocitosis Reactiva

El término gliosis reactiva se usa para la respuesta positiva de astrocitos, microglia y células NG2 a diferentes injurias al sistema nervioso.

La astrogliosis reactiva es un conjunto de cambios moleculares, celulares y funcionales en los astrocitos, en respuesta a lesiones sobre el SNC; estas

alteraciones varían de acuerdo con la gravedad de la enfermedad y se regulan a través de moléculas de señalización intra e intercelulares de una manera específica [87].

Los astrocitos se vuelven reactivos en respuesta a prácticamente todas las situaciones patológicas en el cerebro, como axotomía, isquemia, infección y enfermedades neurodegenerativas. Esta reactividad de los astrocitos se caracterizó, en un primer momento, sólo por cambios morfológicos (hipertrofia, remodelación de procesos) y la sobreexpresión de la proteína GFAP. Pero actualmente se conoce que la reactividad implica cambios funcionales, morfológicos y transcripcionales [123].

Aunque los astrocitos se vuelven reactivos en respuesta a toda lesión o enfermedad en el sistema nervioso, el grado de activación de los astrocitos varía [124].

La astrogliosis leve a moderada no se ve diferente de los astrocitos en el tejido sano del SNC. Sin embargo, en el extremo severo de la astrogliosis, los astrocitos reactivos invaden dominios individuales, y la proliferación de astrocitos interrumpe aún más la arquitectura del dominio, con la formación de una cicatriz glial en los casos más extremos [121].

En ciertas condiciones patológicas, los astrocitos reactivos se agregan en el sitio de la lesión, formando una "cicatriz glial". Se pueden hacer distinciones morfológicas entre los astrocitos reactivos formadores de cicatriz glial (que son los astrocitos reactivos alargados en el centro de la lesión) y los astrocitos reactivos hipertrofiados (que se encuentran más lejos del núcleo de la lesión) [125].

Los astrocitos en la mayoría de las neuropatologías entran en un estado reactivo caracterizado por una rápida proliferación, hipertrofia y regulación al alza de las proteínas de filamentos intermedios, como la GFAP [32,112]. Los astrocitos reactivos pueden liberar una amplia variedad de moléculas extracelulares, que pueden ser neuroprotectores, con funciones reparadoras que preservan la homeostasis sináptica (citoquinas como IL-6 y TGF-β), o neurotóxicas, que dan por resultado la pérdida neuronal (IL-1b y TNF-α) [22, 85,126].

La astrogliosis, desde el punto de vista funcional, está dirigida hacia lo siguiente [32]:

a) Aumento de la neuroprotección y soporte trófico de neuronas bajo estrés.
b) Aislamiento del área dañada del resto del tejido del SNC.
c) Reconstrucción de la barrera hematoencefálica comprometida.
d) En la facilitación, posiblemente, de la remodelación de circuitos cerebrales en áreas que rodean la región lesionada.

En cerebros humanos con patología de AD y en modelos animales de AD, se ha encontrado que los astrocitos reactivos se encuentran rodeando los depósitos de Aβ [126].

Los astrocitos reactivos también producen otros múltiples factores neurotróficos para proteger las neuronas, incluido el factor de crecimiento nervioso (NGF), factor neurotrófico ciliar (CNTF), factor básico de crecimiento de fibroblastos (bFGF), BDNF, factor neurotrófico derivado de la glía (GDNF), factor de crecimiento endotelial vascular (VEGF) y eritropoyetina (EPO) [71].

Más recientemente, los astrocitos reactivos se han dividido en dos grupos, A1 y A2, según el tipo de lesión [74].

- La isquemia induce astrocitos A2 reactivos, que expresan niveles elevados de factores neurotróficos y citoquinas tales como CLCF1(cardiotrophin-like cytokine factor 1), LIF (leukemia inhibitory factor), IL-6 y trombospondinas (proteínas con capacidades antiangiogénicas) que sugieren que los astrocitos reactivos A2 son protectores, promoviendo la supervivencia y reparación neuronal.
- La neuroinflamación induce astrocitos A1 reactivos, que regulan positivamente muchos genes de la cascada clásica del complemento (C1r, C1s, C3 y C4), que están implicados en la poda sináptica y conducen a la pérdida de sinapsis, neuronas y oligodendrocitos maduros [127].

4. Astrocitopatía

Astrocitopatía es la disminución en el número de astrocitos, la atrofia/degeneración y la pérdida de función. Puede ocurrir como causa primaria de una enfermedad o como un factor que contribuye al desarrollo y progresión de una enfermedad en particular [92].

La atrofia astroglial, manifestada por la pérdida de función, contribuye a la progresión patológica de una sorprendente variedad de trastornos neurológicos.

Las modificaciones en el número de astrocitos o en su morfología, también pueden ir acompañadas de la liberación de citoquinas inflamatorias y factores neurotóxicos asociados con el estrés oxidativo [40].

La astrocitopatía es característica de los trastornos neuropsiquiátricos, como la demencia frontotemporal, el estrés, el trastorno depresivo mayor, la esquizofrenia y los trastornos por consumo de sustancias [121].

La disminución de los astrocitos y la atrofia astroglial en la esquizofrenia, en la epilepsia del lóbulo temporal y en los principales trastornos depresivos, se considera uno de los mecanismos principales de la conectividad sináptica anormal en estos trastornos psiquiátricos importantes [32, 83].

Los astrocitos atróficos son más pequeños, no envuelven la sinapsis con tanta fuerza y tienen una expresión reducida de GFAP, AQP4 (Aquaporin-4) y GLT-1 [32]. Las consecuencias de estos cambios morfológicos y bioquímicos incluyen la incapacidad de mediar la homeostasis del glutamato en la sinapsis, el tono reducido de las moléculas de señalización y factores tróficos, debido a la distribución de volumen y producción reducida, metabolismo de glucosa alterado y conectividad de red reducida de los astrocitos [128].

La evidencia reciente asigna a las disfunciones de astrocitos un papel crítico en el envejecimiento y en varias enfermedades neurodegenerativas, incluida la enfermedad de Alzheimer [129].

En el cuerpo estriado isquémico, el número de astrocitos aumenta después del accidente cerebrovascular, y los largos procesos de astrocitos forman una red que une la SVZ y el cuerpo estriado isquémico. Estos astrocitos pueden contribuir a guiar a los neuroblastos que migran al área del cerebro infartada [52].

En la esquizofrenia, aunque no se observan cambios en el nivel de expresión de GFAP, un estudio encontró una regulación positiva masiva de las proteínas de la matriz extracelular en los astrocitos de la corteza entorrinal de pacientes esquizofrénicos, sugiriendo una alteración en la función astrocítica [69].

Bajo condiciones patológicas, los astrocitos hiperreactivos exacerban sus funciones heterogéneas, presentando un rol opuesto que contribuye al desequilibrio del sistema nervioso central (SNC) [85].

En general, se considera que ciertos niveles de gliosis después de la lesión pueden ser beneficiosos para el proceso de recuperación después de una lesión cerebral, mientras que la gliosis excesiva y sus respuestas neuroinflamatorias asociadas tendrán un impacto negativo en la recuperación estructural y funcional del cerebro [102].

G. ESTRÉS

Hans Selye (1936) introdujo por primera vez el concepto de estrés como "la respuesta no específica del cuerpo a cualquier demanda", y desde esa época se han realizado muchos intentos por refinar su significado [130].

El estrés es un evento que amenaza la homeostasis del organismo y como resultado causa respuestas fisiológicas y conductuales que intentan restablecer el equilibrio [107].

Es importante resaltar que los distintos tipos de estrés requieren diferentes respuestas por parte de nuestro organismo. Los factores estresantes físicos como el trauma, frío o la hemorragia sanguínea, requieren una inmediata participación activa del tronco encefálico e hipotálamo; mientras que los estresantes psicológicos comprometen mediadores del estrés en zonas que favorecen la emoción (amígdala y corteza prefrontal), aprendizaje y memoria (hipocampo) y la toma de decisiones (corteza prefrontal) [131].

Si la respuesta al estrés es inadecuada, en intensidad o duración, puede provocar una sobrecarga alostática (distrés) de prácticamente cualquier órgano (incluido el cerebro) y comprometer sus funciones. Estas alteraciones pueden estar directamente asociadas con cambios neuropatológicos en el cerebro [132].

El impacto del estrés sobre los procesos cognitivos y específicamente la memoria, es el énfasis del estudio de la glía en los cambios inducidos por el estrés. Los astrocitos, microglia y oligodendrocitos tienen participación única en el aprendizaje y la memoria. Estos tres tipos celulares expresan receptores tanto para la norepinefrina como para los glucocorticoides y, por lo tanto, son blanco de las acciones de la hormona del estrés [133].

El hipocampo, en modelos animales, ha sido objeto de mucho estudio para dilucidar los efectos del estrés y los trastornos relacionados con el estrés ocurridos en el cerebro humano [134].

Tanto el estrés agudo como el crónico afectan profundamente el aprendizaje y la memoria dependiente del hipocampo: el estrés moderado generalmente mejora los procesos, mientras que el estrés crónico o extremo puede afectar los procesos neurales y cognitivos.

Innumerables estudios en animales han revelado una disminución de las neuronas hipocampales, cuando han sido expuestos a estrés [135].

Las tres etapas de la neurogénesis adulta, proliferación celular, diferenciación neuronal y supervivencia celular pueden verse influidas por el estrés, el aprendizaje

y el enriquecimiento ambiental, pero la mayoría de la evidencia apunta a los efectos del estrés sobre la proliferación celular [30].

Las dendritas o espinas en el hipocampo (y otras regiones) se alteran por el estrés a través de mecanismos intracelulares que reorganizan el citoesqueleto neuronal, produciendo cambios estructurales en actina y microtúbulos [136].

Los astrocitos son las células gliales más estudiadas en relación al efecto del estrés sobre la memoria y el aprendizaje.

El estrés crónico está asociado con una disminución notable del volumen hipocampal y de la corteza prefrontal, observándose una disminución importante de los astrocitos más que del tejido neuronal. En modelos animales, las investigaciones realizadas por Lambert (2000) y Jauregui-Huerta (2010), mostraron incrementos del volumen de astrocitos en estrés de corta duración y disminución del volumen en estrés crónico [133].

Contrario al estrés agudo, el estrés impredecible repetido o prolongado causa un déficit muy importante en la memoria de trabajo y memoria de reconocimiento [137].

El estrés es el mayor factor de riesgo que predispone a los trastornos neuropsiquiátricos, incluida la depresión, la ansiedad, el trastorno de estrés postraumático y la esquizofrenia [138].

El estrés también puede afectar una gran cantidad de factores de crecimiento involucrados en la memoria y el aprendizaje.

El estrés agudo y crónico puede tener efectos muy diferentes sobre el aprendizaje: mientras que el estrés agudo puede potenciar el aprendizaje, el estrés crónico conduce a déficits en la memoria dependiente del hipocampo que recuerdan a la depresión mayor [119].

Los estudios post mortem de pacientes con trastornos psiquiátricos relacionados con el estrés han demostrado una disminución en el número de astrocitos y el nivel de GFAP [139]. En modelos de ratón se ha evidenciado que la eliminación de GFAP podría disminuir la resistencia del tejido cerebral al estrés mecánico severo [98].

En roedores, el estrés disminuye la neurogénesis; mientras que los antidepresivos, el enriquecimiento ambiental, el ejercicio y el aprendizaje estimulan la neurogénesis [109].

Es importante destacar que el resultado del estrés parece estar determinado por la duración y la gravedad del factor estresante. En los seres humanos, el deterioro de la función cognitiva también se correlaciona con la duración del factor estresante [140].

La investigación sobre la neurobiología de la respuesta al estrés en animales ha dado lugar a nuevos tratamientos exitosos para el trastorno de estrés postraumático (Posttraumatic stress disorder, PTSD) en los seres humanos. La exposición crónica al estrés conduce a la atrofia dendrítica en PFC, la extensión dendrítica en la amígdala y el fortalecimiento del sistema noradrenérgico [141].

El estrés también estimula la producción y liberación de norepinefrina (NE) al causar la secreción incrementada de factor liberador de corticotropina (CRF) del hipotálamo, que a su vez desencadena la liberación de ACTH de la glándula pituitaria, que posteriormente estimula a la glándula suprarrenal para liberar NE y cortisol. Los niveles incrementados de cortisol y NE posteriormente aumentan el impulso simpático y la liberación de citoquinas, que se ha demostrado que tienen efectos recíprocos en el eje HPA, así como efectos neurotóxicos [15].

La desregulación del eje HPA es una característica común de la depresión y la alteración de la morfología neuronal del hipocampo juega un papel primordial en la desregulación de la liberación de glucocorticoides, controlados por el eje HPA, que resulta de la exposición al estrés [19].

El estrés induce alteración de varias vías de transducción de señales que incluyen cAMP-PKACREB, cAMP-ERK1 / 2-CREB, cAMP-PKA, Ras-ERK, PI3K-Akt, TNFα-NFκB, GSK-3β, mTOR y CREB y que también se asocia con la pérdida o aumento crónico de la espina dendrítica o arborización en ciertas áreas del cerebro [113, 134].

En otro aspecto, los déficits de cognición inducidos por el estrés crónico se atribuyen a modificaciones epigenéticas tales como: el aumento en la acetilación y metilación de las histonas [142].

Es importante señalar que las neuronas y las células gliales, tienen una gran demanda de O_2, para producir la energía necesaria para mantener su homeostasis iónica y la síntesis de neurotransmisores, entre otras funciones. En condiciones fisiológicas, entre un 1% al 2% del O_2 consumido por las mitocondrias se convierte normalmente en especies reactivas de oxígeno (ROS), como el anión superóxido (O_2^-) y el peróxido de hidrógeno (H_2O_2).

Siguiendo la idea anterior, el estrés oxidativo -que se define como una condición en la cual la generación de estas especies abruma las defensas antioxidantes- está ampliamente implicado en la patogénesis de muchas enfermedades neurológicas y psiquiátricas [143].

1. Aspecto Neuroendocrino del Estrés

Dos sistemas neuroendocrinos principales se activan en respuesta a factores estresantes, el sistema simpáticoadrenal (SAS) y el eje HPA. La activación de SAS induce predominantemente la redistribución del flujo sanguíneo al cuerpo y la activación del eje HPA es principalmente responsable de la regulación del metabolismo [132].

La exposición a un estresor produce un aumento inmediato de catecolaminas a través de la activación de neuronas preganglionares simpáticas en la médula espinal [144].

La hormona liberadora de corticotrofina y la vasopresina son los motores principales del sistema hormonal del estrés, provocan la liberación de la corticotropina (ACTH) hacia la periferia y, por lo tanto, activan la síntesis de corticosteroides en la corteza suprarrenal. Si estas hormonas del estrés son persistentemente hipersecretadas, pueden provocar condiciones clínicas graves y obstaculizar una adaptación adecuada al estrés. Estas hormonas también actúan como neuromoduladores en el cerebro, afectando las funciones mentales superiores, incluidas la emoción, la cognición y el comportamiento [145].

En general, un aumento de la corticosterona debido al estrés crónico puede ser una causa de perturbación en la formación de la memoria [71]. El exceso de glucocorticoides aumenta la liberación de glutamato en la región CA1 del hipocampo, y el estrés conductual crónico aumenta los niveles extracelulares de glutamato en la región CA3 [119].

Tres áreas cerebrales que se han visto alteradas por el aumento de glucocorticoides son la corteza prefrontal medial (mPFC), el hipocampo y la amígdala. La mPFC está involucrada en el funcionamiento ejecutivo y el procesamiento de la emoción, el hipocampo está involucrado en la memoria y el aprendizaje, y la amígdala está involucrada en el procesamiento de la emoción [15].

El estrés crónico y el tratamiento con corticosterona afectan la morfología dendrítica de las áreas límbicas del cerebro de la rata, como el hipocampo, el complejo amigdaloide y la corteza prefrontal. Estas alteraciones aumentan la ansiedad y perjudican la memoria y el aprendizaje espacial [146]. Se ha demostrado un aumento en la liberación de monoaminas después del estrés en el hipocampo, la amígdala, la corteza prefrontal y el núcleo accumbens, pero probablemente también tiene lugar en muchas otras regiones del cerebro.

Se ha demostrado que el estrés crónico disminuye la complejidad dendrítica de las neuronas piramidales y aumenta la actividad transcripcional de las interneuronas GABA en el mPFC, disminuyendo la actividad en esta área [147].

Los glucocorticoides (GC) son potentes moduladores y reguladores de los procesos de memoria del hipocampo. Los aumentos moderados en los GC pueden mejorar los procesos cognitivos, pero las concentraciones muy elevadas de los GC alteran de forma aguda la función del hipocampo. Los niveles persistentemente elevados de glucocorticoides durante el estrés crónico pueden reducir la plasticidad sináptica y el número de neuronas en el hipocampo. Este efecto está mediado por varios mecanismos, incluida la atenuación de la expresión de BDNF [132].

El aumento de los niveles de cortisol afecta la capacidad del hipocampo para adaptarse a un entorno cambiante. Los animales con estrés crónico presentan plasticidad y LTP reducidos en las neuronas de la región CA3 del hipocampo, mediadas por el receptor de glucocorticoides (GR), lo que conduce a una adaptación y aprendizaje deficientes [148].

Los considerables datos experimentales sugieren que los GC son los principales efectores del daño del hipocampo causado por diferentes tipos de estrés [149].

Los glucocorticoides reducen la plasticidad sináptica mediante la activación del glucógeno sintetasa quinasa-3 (GSK-3), que induce el debilitamiento sináptico por fosforilación de la proteína Tau [150].

También se sugiere que la atrofia del hipocampo inducida por los corticosteroides puede jugar un papel importante en la patogénesis de una variedad de trastornos neuropsiquiátricos [149].

Las monoaminas promueven colectivamente importantes estrategias de comportamiento que ayudan al animal a enfrentar y sobrevivir a la fase inicial de un evento estresante. La noradrenalina nos permite enfocar nuestra atención, desde la información sensorial a una exploración más general del entorno, lo que puede proporcionar mejores soluciones para condiciones desafiantes.

Se cree también que la dopamina, liberada durante el estrés moderado en la corteza prefrontal, mejora la evaluación del riesgo y la toma de decisiones, y la serotonina, que es fundamental para reducir la ansiedad posterior al estrés [131].

2. Estrés Agudo

El estrés agudo incluye muchos mecanismos de tipo adaptativos imprescindibles para la supervivencia, por su parte el estrés crónico induce una activación excesiva y disfunción de los sistemas activados por estrés, resultando en daño cerebral adicional y comportamiento de tipo depresivo [151].

El estrés agudo incluye todas las respuestas fisiológicas y / o psicológicas a eventos que requieren un ajuste conductual para superarlos.

Revisando varios estudios en humanos, se puede destacar que el estrés agudo mejora significativamente el rendimiento de la memoria de trabajo en ratas jóvenes y también, puede mejorar la capacidad posterior de adquirir nueva memoria asociativa [152, 153].

Algunos estudios también muestran que el estrés agudo e incontrolable afecta las funciones cognitivas mediadas por la corteza prefrontal [137].

La administración oral de hidrocortisona mejora el rendimiento de la memoria operativa y eleva la actividad de la PFC dorsolateral en humanos [137].

3. Estrés Crónico

El estrés crónico se considera un factor clave en el desarrollo de diversas enfermedades somáticas y neuropsiquiátricas [132].

Si bien es cierto que la respuesta al estrés agudo es un proceso necesario para adaptarse a los cambios ambientales que ocurren durante la vida (para un afrontamiento efectivo); el estrés severo o crónico, puede resultar en un factor de riesgo para el desarrollo de varios trastornos psiquiátricos como depresión y trastorno de estrés postraumático (PTSD) [107].

Las alteraciones neuropsiquiátricas son los efectos más ampliamente descritos de la exposición crónica al estrés e incluyen el comportamiento de tipo ansioso, el comportamiento depresivo y los déficits cognitivos.

No obstante, los efectos del estrés crónico no sólo se limitan a cambios de comportamiento. Las células inmunes expresan receptores para glucocorticoides y catecolaminas, que pueden conducir a alteraciones en la transcripción génica en respuesta al estrés [154, 155].

El estrés puede alterar la señalización neuronal de múltiples maneras. Las observaciones dispersas en la literatura sugieren que el estrés crónico puede alterar las vías de señalización implicadas en la plasticidad sináptica [119].

Los estudios en roedores han demostrado que la exposición sostenida al estrés induce la pérdida de dendritas y espinas en el PFC. La pérdida de espinas y / o dendritas se correlaciona con una memoria de trabajo deteriorada, una menor flexibilidad atencional [141], coincidentes con los déficits de memoria espacial dependientes del hipocampo [136] y trastornos similares a la depresión.

El estrés crónico produce cambios consistentes en el hipocampo que incluyen la disminución de la longitud dendrítica, la reducción del número de ramas y la disminución de la expresión de NCAM (neural cell adhesion molecule) [149].

El estrés crónico puede afectar la función del hipocampo a través de mecanismos como la remodelación neuronal en el área CA3, la supresión de la actividad sináptica y la neurogénesis alterada [156].

En ratones C57B/6, 10 días de estrés por inmovilización crónica indujeron la retracción dendrítica de neuronas piramidales CA3 de eje corto, pero no de neuronas piramidales de eje largo CA3, junto con una retracción robusta de dendritas en neuronas piramidales CA1 dorsal [134].

El estrés crónico produce una disminución selectiva en la proliferación celular y reducción en las células inmunoreactivas a GFAP en la corteza prefrontal y en el giro dentado del hipocampo [157].

El estrés crónico puede activar la astroglia, con una mayor síntesis de GFAP o causar atrofia, con menos procesos y menos GFAP, como en los cerebros de ratas bajo estrés crónico impredecible o psicosocial [158, 159].

Analizando lo comentado hasta ahora, el impacto del estrés o las hormonas relacionadas con la regulación astroglial aún no está claro y existe escasa información sobre los mecanismos que mejoran o perjudican la función de los astrocitos en el cerebro expuesto a estrés.

Se cree que el estrés crónico altera el sistema noradrenérgico, que está íntegramente relacionado con los sistemas neuroendocrino e inmunitario. Por ejemplo, el estrés crónico conduce a un aumento en la actividad de la tirosina hidroxilasa, la enzima involucrada en la síntesis de NE, en el locus ceruleus [15].

Algunas conclusiones observadas en distintos trabajos dan cuenta que el estrés crónico aumenta el crecimiento dendrítico en la amígdala, lo que acentúa el desequilibrio de la amígdala sobre la función de la corteza prefrontal [141].

El estrés crónico en humanos también debilita la conectividad funcional de la PFC y la regulación de la PFC sobre la amígdala. Dado lo anterior, la exposición sostenida al estrés conduce a cambios más persistentes en los circuitos cerebrales que regulan el comportamiento y la emoción, manteniendo el cerebro en un estado más primitivo y reactivo [141, 160].

También disminuye la LTP en las proyecciones desde la amígdala basolateral hasta la mPFC y aumenta la excitabilidad en la amígdala, en respuesta al estrés, lo que resulta en una mayor reactividad al estrés y disminución del procesamiento cognitivo [15].

El estrés social crónico disminuye la proliferación celular en las ratas y los ratones, donde el comportamiento subordinado se correlaciona negativamente con las tasas de proliferación celular [30].

El estrés crónico también cambia la memoria funcional, del aprendizaje basado en el hipocampo al aprendizaje habitual basado en striatum. Ya que el efecto de los glucocorticoides, mediado por el receptor de mineralocorticoides (MR), es provocar un desacoplamiento de la amígdala del hipocampo y producir una mayor conectividad de la amígdala al estriado [161].

Se ha demostrado que el estrés leve crónico causa la sobreexpresión del óxido nítrico sintetasa (NOS) del hipocampo y la producción resultante de óxido nítrico (NO) se ha visto implicada en la etiología de la depresión [162].

4. Estrés por Restricción Crónica

Los modelos de estrés de roedores son ampliamente utilizados para investigar la base neurobiológica de los trastornos psiquiátricos. El uso de modelos de estrés está respaldado por evidencia sustancial que implica al estrés como un factor desencadenante de varios trastornos neuropsiquiátricos [163].

En vista de las observaciones clínicas de que el estrés precipita la depresión y que la enfermedad depresiva recurrente está asociada con la contracción del volumen del hipocampo, el estrés crónico puede servir como modelo experimental para evaluar las alteraciones celulares y moleculares subyacentes asociadas con algunas de las consecuencias de la enfermedad depresiva recurrente [164].

Los modelos animales de estados psiquiátricos son procedimientos aplicados a animales de laboratorio que engendran cambios de comportamiento que pretenden ser homólogos a aspectos de trastornos psiquiátricos, por lo tanto, pueden usarse como herramientas experimentales para ampliar la comprensión de la psicopatología humana [165].

En el modelo crónico de estrés leve (CMS) de depresión, las ratas o ratones están expuestos crónicamente a un bombardeo constante de microesfuerzos impredecibles, lo que resulta en el desarrollo de una plétora de cambios de comportamiento, incluida una menor respuesta a las recompensas, un correlato conductual del síntoma central clínico de la depresión, la anhedonia [166].

El estrés de restricción (restraint stress, RS) es un modelo de estrés crónico en roedores que promueve la remodelación neuronal de regiones límbicas, como también las células neuronales de la PFC, CA1 y CA3 del hipocampo. RS también induce deterioro de la memoria de trabajo, ansiedad y comportamiento de tipo

depresivo. Estas anomalías se asemejan a las observadas en humanos expuestos a estrés crónico o estrés agudo [167, 168].

El estrés de restricción afecta la memoria espacial dependiente del hipocampo y la LTP del hipocampo. Dichos efectos se han asociado a la retracción de las dendritas apicales y a la pérdida de sinapsis en la subregión CA3 del hipocampo [151]. Las dendritas CA3 son más sensibles al estrés crónico que las dendritas CA1 [113].

Un estrés de restricción de movimiento por 21 días produce deterioro de la ramificación dendrítica, atrofia y pérdida de las espinas dendríticas en las neuronas piramidales de la PFC [137].

Un grupo de investigación dio a conocer que un protocolo de 10 días de inmovilización, produjo significativas alteraciones en la arborización dendrítica y densidad espinal en la corteza frontal ventromedial, amígdala basolateral y en la región CA3 del hipocampo en ratas y ratones [169].

También se ha demostrado que el estrés por restricción crónica reduce la supervivencia de nuevas neuronas en ratas, pero mejora la supervivencia de nuevas neuronas en ratones. El modelo de estrés en base a descarga eléctrica crónica disminuye la proliferación celular y la diferenciación neuronal en ratas adultas [30].

Aunque varios estudios han sugerido que el aprendizaje aumenta la neurogénesis en la circunvolución dentada adulta, cuando el aprendizaje es difícil o estresante, puede tener un impacto negativo en la proliferación celular [170].

Imbe et al., 2013, estudiando el efecto del estrés por restricción sobre el tejido glial, utilizando un modelo animal de estrés por restricción crónica, encontraron reducción del nivel de proteína GFAP en el bulbo rostral ventromedial (RVM). Concluyeron que este hallazgo sugiere que la disminución de la proteína GFAP, inducida por el estrés de restricción crónica, causa la disfunción de los astrocitos, que pueden estar implicados en el deterioro del RVM (que desempeña un papel fundamental en la modulación del dolor) [121, 171].

Se ha demostrado que el estrés por restricción crónica disminuye el GFAP en el hipocampo y la corteza frontal, RVM y la sustancia gris periacueductal [171].

El estrés por restricción incrementa los niveles extracelulares de glutamato [164].

En el paradigma de restricción crónica, los animales se colocan en un dispositivo de restricción (bolsa o tubo, ventilados adecuadamente), durante 1 a 6 horas, por un período de 14-21 días o más [113]. Esta restricción del movimiento implica un componente físico (inmovilización), pero actúa principalmente como un factor estresante psicológico a través de la conciencia de la incapacidad para escapar [156].

El modelo de estrés por restricción crónica (CRS) se ha utilizado ampliamente para estudiar la alteración morfológica, hormonal y del comportamiento, en varias regiones cerebrales de roedores, como el hipocampo, la corteza prefrontal, la amígdala y el núcleo accumbens, porque es económico y relativamente fácil de implementar.

En los seres humanos, el estrés crónico o el estrés psicosocial también produce atrofia del volumen del hipocampo y cambios funcionales en la corteza prefrontal [146].

Además de inducir la retracción dendrítica del hipocampo, el estrés por restricción crónica también se ha usado ampliamente para evaluar otras propiedades del hipocampo, incluida la expresión molecular, la actividad sináptica y comportamientos dependientes del hipocampo, como la memoria espacial [156].

Dependiendo de la duración e intensidad del estrés crónico, algunos estudios informan que la exposición de los animales a CRS induce comportamientos similares a la depresión, como la anhedonia [113].

CRS de 21 días o más causa cambios tales como atrofia dendrítica y deterioro de la memoria [113].

También se ha observado en humanos, pérdida de materia gris de la corteza prefrontal, bajo estrés crónico [172].

5. Cambios Morfológicos por el Estrés

El estrés crónico causa atrofia dendrítica y pérdida de espinas dendríticas en las áreas CA1 y CA3 del hipocampo, y también reduce la generación de nuevas neuronas en el giro dentado.

Debido a lo anterior, el estrés crónico reduce el BDNF, un regulador clave del crecimiento neuronal y la plasticidad morfológica. La fisiología sináptica también se ve afectada, y se caracteriza por el deterioro de la LTP, un mecanismo de plasticidad sináptica que se considera crucial para el aprendizaje y la memoria [106].

El estrés disminuye el BDNF en el hipocampo y otras regiones límbicas del cerebro, lo que puede contribuir a la atrofia de dendritas en las neuronas hipocampales [16]. Afectando finalmente la neurogénesis del hipocampo adulto.

Como se mencionó más arriba, se ha demostrado que el estrés provoca la pérdida de espinas dendríticas en la región CA3 del hipocampo, aunque también se ha informado de un aumento o ausencia de cambios en la densidad de la espina dendrítica [167].

Debido a que el estrés tiene efectos negativos sobre el crecimiento de neuritas y la formación de sinapsis, se cree que los cambios neuronales resultantes afectan el comportamiento y se considera que juegan un papel importante en los trastornos psiquiátricos [173].

La pérdida de espinas dendríticas y la retracción de las dendritas apicales de las neuronas piramidales del hipocampo CA3 se han documentado en animales crónicamente estresados y tejido post mortem de sujetos deprimidos, pero los efectos del estrés crónico y la depresión en las neuronas piramidales CA1 ha sido menos caracterizado [17].

Por lo tanto, los cambios inducidos por el estrés en las neuronas CA3 del hipocampo son consistentes con los déficits en la función del hipocampo, incluida la memoria espacial [156].

El DG experimenta una reducción del número de células bajo estrés crónico y en respuesta a los niveles elevados de corticosterona; mientras que la actividad física y un entorno enriquecido, aumentan el número de neuronas y el volumen del giro dentado.

Los estudios también han sugerido la atrofia y disminución de la expresión de GFAP, en lugar de reducciones en el número de astrocitos en el estrés relacionado con enfermedades neurodegenerativas. La implicación de la señalización del factor de crecimiento de fibroblastos (FGF) también se ha demostrado en la morfología alterada de astrocitos durante la activación glial [51].

Se detectó una disminución del número de perfiles positivos de GFAP astrogliales y de la inmunorreactividad global de GFAP en los modelos animales de estrés crónico [2].

El estrés de la vida temprana en forma de privación posnatal temprana condujo a astrocitos GFAP+ muy reducidos en regiones del cerebro asociadas con el comportamiento relacionado con el estrés, incluyendo la corteza prefrontal, la corteza cingulada, la amígdala y ciertas regiones del hipocampo [69].

El impacto del estrés sobre la neurogénesis adulta es de particular interés porque la inhibición de la neurogénesis adulta afecta el comportamiento ansioso depresivo inducido por el estrés en roedores y se sugiere que también desempeña un papel en la depresión en humanos [174].

6. Cambios Moleculares por el Estrés

Varios efectos que la glía ejerce sobre la memoria, bajo estrés, son mediados a través de reacciones neuroinflamatorias [175], existiendo a la vez interacción

bidireccional entre la glía y las hormonas del estrés, por ejemplo, moléculas como la interleukina 1 (IL-1) e interleukina 6 (IL-6), liberadas por la glía, pueden activar el eje hipotálamo-pituitaria-adrenal (HPA) [176].

Mientras que el efecto potenciador del estrés agudo está mediado principalmente por los glucocorticoides que activan las terminaciones nerviosas glutamatérgicas presinápticas, el estrés crónico ejerce su efecto muy probablemente mediante la alteración de la regulación de la liberación de glutamato [132, 133].

Los cambios en la liberación presináptica de glutamato, el tráfico y expresión del receptor de glutamato postsináptico, la estructura de las espinas sinápticas, el citoesqueleto y el control epigenético de los genes de la plasticidad contribuyen a los efectos complejos del estrés [137].

La introducción de un corto período de estímulos estresantes para los animales, como natación forzada, breve y bajo choque eléctrico en las patas o restricción del movimiento, producen una transmisión glutamatérgica significativamente mejorada en los circuitos de PFC.

Existen también correlaciones significativas entre el volumen del hipocampo y el número total de astrocitos positivos para GFAP, así como entre el volumen del hipocampo y los volúmenes somáticos de los astrocitos en animales estresados, sugiriendo que la patología astrocítica da cuenta de la reducción en el volumen del hipocampo [18, 177].

Los estudios preclínicos también demuestran que el estrés disminuye la GFAP y los astrocitos marcados, así como la proliferación de oligodendrocitos [178].

La expresión de BDNF se suprime en animales expuestos a diversos paradigmas de estrés. La aplicación de corticosterona disminuye la expresión de BDNF, pero aumenta el BDNF en animales sometidos a adrenalectomía [179, 180, 181].

El estrés crónico aumenta los niveles de la hormona del estrés glucocorticoide y suprime la producción de nuevas neuronas en el hipocampo. Esta respuesta da como resultado la disminución de la densidad de la espina dendrítica y el número de sinapsis, además de disminución de la memoria [113].

Tanto el estrés agudo como el crónico conducen a reducciones en los niveles de mRNA de BDNF hipocampal, lo que sugiere un deterioro de algunos de los mecanismos de neuroplasticidad. Los glucocorticoides también suprimen la expresión de BDNF [119].

La sobreexpresión de BDNF aumenta la arborización dendrítica en las neuronas del hipocampo, bloquea la atrofia hipocampal inducida por estrés crónico y mejora los comportamientos similares a la depresión. El tratamiento antidepresivo revierte la

regulación negativa de la neurogénesis inducida por el estrés, que es probable a través de la transducción de señal regulada por tirosina quinasa mediada por BDNF [182,183]. La expresión de BDNF está críticamente involucrada en la fase de consolidación de la memoria a largo plazo [142].

Los animales expuestos a estrés crónico impredecible tienen una expresión disminuida de Neuritin, un gen dependiente de la actividad sináptica, que se revierte con el tratamiento antidepresivo. La disminución de Neurtitin previene la atrofia inducida por el estrés de las dendritas y las espinas y los comportamientos similares a la depresión. Otra molécula activada por estrés, mTORC (diana de rapamicina en células de mamífero), también recibe mucha atención en este campo de estudio [137].

7. Estrés y Depresión

La relación entre el estrés y las enfermedades psiquiátricas ha sido bien establecida durante 20 años en la clínica [113].

El estrés y la depresión están muy relacionados. Por ejemplo, los acontecimientos vitales estresantes pueden precipitar episodios depresivos en individuos vulnerables y el estrés infantil en forma de abuso o negligencia, aumenta el riesgo de depresión más adelante en la vida [15].

El estrés experimentado temprano en la vida es un factor de riesgo bien documentado para la precipitación de enfermedades psiquiátricas, incluidos los trastornos del estado de ánimo y la ansiedad [184].

Numerosos factores determinan el impacto del estrés sobre la estructura cerebral, incluido el tipo (físico o psicológico) y la duración del factor estresante (agudo o crónico), la etapa de desarrollo del animal (recién nacido, adolescente, adulto o anciano), el sexo y antecedentes genéticos del animal, las diferencias individuales en el afrontamiento y los niveles iniciales de comportamiento de ansiedad [134, 136, 138, 185].

El estrés y la depresión están asociados con atrofia neural, caracterizada por pérdida de conexiones sinápticas en regiones corticales y límbicas claves que están implicadas en la depresión. Se cree que esto ocurre en parte a través de la reducción de la expresión y función de los factores de crecimiento, tales como el BDNF en la corteza prefrontal y en el hipocampo [178].

El estrés crónico y la depresión se asocian con alteraciones de los procesos neuronales, incluidas las sinapsis de la espina dendrítica, de una manera dependiente de la región, dando como resultado una conectividad interrumpida de los circuitos cerebrales que contribuye a los síntomas depresivos [19].

En las últimas décadas, se han encontrado varias anormalidades en el eje HPA asociadas con una respuesta hiperactiva al estrés en pacientes deprimidos. Algunas de estas alteraciones incluyen hipersecreción del factor liberador de corticotropina (CRF) del núcleo paraventricular del hipotálamo, alteración de la retroalimentación negativa del eje HPA, aumento del volumen de las glándulas suprarrenales, hipercortisolemia y disminución de la supresión del cortisol en respuesta a la dexametasona [15].

La inhibición farmacológica de la conductancia de unión gap en la corteza prefrontal indujo a la anhedonia, que es uno de los síntomas principales de la depresión. Del mismo modo, la inhibición de los transportadores de glutamato astrogliales también indujo la anhedonia.

El estrés crónico y algunos tratamientos antidepresivos ejercen sus efectos sobre la neurogénesis adulta, específicamente en el hipocampo ventral, el área del hipocampo que desempeña un papel principal en la respuesta al estrés y la emotividad, y el estudio de Wu y Hen, 2014, demostró que los efectos ansiolíticos de la fluoxetina dependen de la neurogénesis en esta área del cerebro [107].

El tratamiento crónico con antidepresivos afectó directamente a la astroglia, aumentando la expresión de receptores y transportadores responsables de la homeostasis del SNC y limitando la liberación de glutamato [2].

Los roedores estresados también tienen un bajo rendimiento en las tareas de aprendizaje espacial dependientes del hipocampo. En conjunto, se cree que estos efectos perjudiciales del estrés a nivel molecular y celular afectan el aprendizaje y la memoria del hipocampo [106].

La memoria y la emoción se rigen por procesos de plasticidad homeostática, que son susceptibles al estrés psicológico. El estrés crónico puede inducir déficits que van desde disturbios emocionales transitorios hasta disfunciones más persistentes de memoria y/o del estado de ánimo, lo que resulta en varios trastornos depresivos y de ansiedad tales como trastorno de estrés postraumático (PTSD) y trastorno depresivo mayor (MDD), entre otros [167].

H. TRASTORNO DEPRESIVO MAYOR (MDD)

El trastorno depresivo mayor (MDD) o depresión mayor es una enfermedad psiquiátrica severa, que afecta a un gran porcentaje de la población y su incidencia va en incremento, con graves consecuencias sociales y económicas [157].

La depresión es un trastorno de salud mental común, con un estimado de 350 millones de personas afectadas [186].

Walter Cannon, prominente fisiólogo de la Universidad de Harvard, durante los primeros años del siglo XX, fue uno de los primeros científicos en vincular los estados emocionales con la actividad suprarrenal [187].

Los síntomas clínicos como baja autoestima, pérdida de interés o placer, fatiga y sentimientos de inutilidad son características de la depresión. Aunque se han logrado grandes mejoras en el tratamiento antidepresivo, nuestro conocimiento de la etiología de la depresión sigue siendo limitado [188].

El comportamiento general de estos individuos se caracteriza por malestar, hiperalgesia, pirexia, desinterés en las interacciones sociales, letargo, inhibición del comportamiento, reducción de la actividad locomotora, exploración y arreglo personal, reducción del rendimiento reproductivo, anhedonia, somnolencia, anorexia y pérdida de peso, falta de concentración y ansiedad.

Es importante destacar que la depresión ocurre en aproximadamente el 35% de los pacientes con enfermedad de Parkinson y podría desarrollarse en la etapa premotora de la enfermedad [189].

Existe amplia evidencia que eventos estresantes de la vida diaria, estrés agudo o crónico, incrementan el riesgo de MDD [151].

Actualmente se reconoce que el déficit de monoaminas no explica, por sí solo, la fisiopatología del MDD, sino que existen otros mecanismos y factores que es importante considerar para el tratamiento de esta enfermedad [190].

Estudios recientes han demostrado que la depresión está estrechamente relacionada con la disminución de la plasticidad estructural, la resiliencia celular alterada y la atrofia neuronal [157]. Las distintas alteraciones de la plasticidad de los astrocitos en el sistema nervioso central están asociadas con MDD [191, 192].

Estudios postmortem recientes también demostraron la densidad sináptica reducida en la corteza prefrontal de los sujetos con depresión [193].

Las personas con depresión tienen un hipocampo significativamente más pequeño que los individuos sanos, lo que puede ser el resultado de una disminución en la arborización dendrítica y la densidad en la columna de las neuronas del hipocampo.

La atrofia hipocampal en pacientes deprimidos se asocia con la gravedad de la depresión [113].

Se ha formulado la hipótesis de que la neurogénesis adulta alterada es parte de la patogénesis del trastorno depresivo mayor. La neurogénesis se ve afectada en los modelos de depresión inducidos por el estrés en roedores [109].

También se ha informado sobre un menor volumen del hipocampo en MDD, junto con apoptosis en células neuronales de la corteza entorrinal, subículo, regiones DG, CA1 y CA4 del hipocampo [194].

En las últimas décadas, la evidencia emergente ha demostrado que el aumento de la neurogénesis en el hipocampo puede mejorar los comportamientos antidepresivos de los animales.

El bloqueo de la neurogénesis del hipocampo a través de la radiación o los métodos genéticos impidió la respuesta de los animales a los antidepresivos. Sin embargo, el aumento de la neurogénesis del hipocampo adulto con modificación genética es suficiente para reducir los comportamientos depresivos y ansiosos [193].

La imagen cerebral y los estudios postmortem han identificado las estructuras clave involucradas en la regulación del estado de ánimo y la depresión, incluida la corteza prefrontal, el hipocampo, la corteza cingulada, la amígdala y los ganglios basales. 5,10 Estudios del flujo sanguíneo y de imágenes funcionales han identificado regiones con actividad reducida (PFC e hipocampo) o actividad aumentada (corteza cingulada subcallosal y amígdala) [178].

Imágenes estructurales han demostrado una disminución del tamaño del hipocampo en pacientes con depresión mayor, especialmente en aquellos que han sufrido múltiples episodios [119]. La mayoría de los hallazgos volumétricos informados concuerdan con la reducción de los volúmenes del hipocampo en sujetos depresivos [145].

Según las pruebas recopiladas durante las últimas dos décadas, las células gliales, particularmente los astrocitos, podrían contribuir a la fisiopatología y la patogénesis del MDD y, por lo tanto, tienen importancia farmacológica como dianas farmacológicas [18, 177, 191].

La creciente evidencia de estudios clínicos, preclínicos y post mortem ha revelado una disminución en el número o densidad de astrocitos y atrofia astroglial morfológica y funcional, en pacientes con MDD y en modelos animales de depresión. Además, los tratamientos antidepresivos actualmente disponibles ejercen, al menos parcialmente, sus efectos terapéuticos sobre los astrocitos [18].

Existe una creciente comprensión de que la patología astroglial, especialmente en las áreas fronto-límbicas del cerebro puede contribuir sustancialmente a la fisiopatología de los trastornos del estado de ánimo. El análisis morfométrico ha revelado cambios mucho más pronunciados en el número de células gliales (astrocitos y oligodendrocitos) que, en el número de neuronas, en el contexto de los trastornos del estado de ánimo, trastornos depresivos y bipolares [2, 177, 179, 180].

La pérdida de espinas neuronales, inducida por estrés, se ha demostrado en CA1 (y CA3) junto con una transmisión sináptica alterada y comportamientos depresivos. Disminución de la densidad de espinas en las neuronas CA1 se ha asociado con comportamientos similares a la depresión en un modelo de depresión inducida por la luz, en ausencia de cambios en las espinas de la región CA3 o cambios dendríticos en las regiones CA1 o CA3 [19].

Muchas otras funciones astrocíticas se alteran en la depresión mayor, incluida la homeostasis de iones y agua, el reciclaje del neurotransmisor GABA y monoaminas, la integridad de la barrera hematoencefálica, la gliogénesis y la sinaptogénesis [18].

La activación del eje HPA es un sello distintivo de la depresión mayor [113] y el deterioro cognitivo es el endofenotipo central de la depresión mayor [119].

Entre los hallazgos de la estructura cerebral alterada y la función en la depresión, el más consistente es el volumen reducido de la PFC e hipocampo. El volumen reducido se correlaciona inversamente con la duración de la enfermedad, el tiempo de tratamiento y la gravedad de la depresión [178].

El estrés psicológico es el factor desencadenante más común de los trastornos del estado de ánimo. Dado que el estrés activa GSK3 (serine/threonine protein kinase) y causa neuroinflamación, un objetivo actual de las investigaciones es descifrar las interacciones entre el estrés, la activación de GSK3 y la inflamación, para determinar si pueden proporcionar nuevas vías para intervenir en los trastornos del estado de ánimo [195].

Estudios detallados de modelos preclínicos de depresión han proporcionado una amplia evidencia que demuestra que el estrés crónico causa alteraciones de la densidad y la función de las sinapsis de la espina sináptica en regiones cerebrales claves, como las zonas límbicas y corticales (implicadas en la depresión) [19].

Una hipótesis importante para la fisiopatología de la depresión, la hipótesis de las monoaminas, postula que la depresión es causada por una alteración en los niveles de una o más de las monoaminas, que incluyen serotonina (5-HT), norepinefrina (NE) y dopamina (DA). La evidencia de la teoría serotoninérgica incluye el hallazgo de que los metabolitos de serotonina se reducen en pacientes diagnosticados con MDD y que los antidepresivos, como los antidepresivos tricíclicos (TCA), los

inhibidores selectivos de la recaptación de serotonina (ISRS) y los inhibidores de la recaptación de serotonina y norepinefrina (IRSN), aumentan los niveles de serotonina en el cerebro [15].

Los cambios informados en la expresión de GFAP relacionados con los trastornos del estado de ánimo que incluyen depresión, estrés y ansiedad han sido notablemente consistentes.

En el tejido cerebral post mortem de pacientes diagnosticados con MDD, se ha encontrado una disminución constante en la expresión de GFAP en diversas regiones cerebrales. Se han encontrado reducciones en GFAP en la corteza prefrontal, corteza prefrontal dorsolateral y corteza orbitofrontal [121].

La falta de GFAP en ratones con deficiencia de GFAP redujo el número de transportadores de glutamato, tanto en neuronas como en astrocitos, una disminución que probablemente modifique la fuerza sináptica [7].

Se ha reportado una reducción en la expresión del marcador de astrocitos, GFAP, en el tejido cerebral post mortem de sujetos deprimidos. Los estudios en animales también revelaron reducciones en el GFAP hipocampal después de la exposición al estresor que se correlacionaba con comportamientos de tipo depresivo [196].

Existe una reducción en el número de células gliales en la amígdala, la corteza cingulada anterior (ACC) y en la PFC de las personas con depresión mayor [197].

El metabolismo energético alterado en los astrocitos da lugar a la atrofia de los astrocitos, afectando todas las funciones astrocíticas y, finalmente, induciendo una falla de la homeostasis neuronal en regiones cerebrales, que incluyen el hipocampo y la PFC, que son responsables de la expresión de fenotipos similares a la depresión central como anhedonia y desesperanza [18].

La inflamación y la exposición al estrés generalmente se reconocen como factores potentes para el desarrollo de síntomas de depresión [189].

Las citoquinas proinflamatorias, que son elevadas durante los trastornos de la depresión, pueden estimular el eje hipotalámico-pituitario-adrenal para liberar los glucocorticoides y, por consiguiente, inducir múltiples manifestaciones relacionadas a la depresión. Se han reportado niveles crecientes de citoquinas pro-inflamatorias tales como las IL-1, IL-6, IL-8, IL-12, interferón-γ y el factor de necrosis tumoral (TNFα) en pacientes con depresión clínica [193].

La inflamación en pacientes con depresión está asociada con la resistencia al tratamiento con fármacos antidepresivos clásicos, y existe cierta evidencia de que los fármacos antiinflamatorios pueden mejorar las acciones antidepresivas [195].

Una amplia evidencia indica que el BDNF, desempeña un papel primordial en la plasticidad sináptica y en el establecimiento de la memoria a largo plazo, pudiendo también desempeñar un papel crítico en el desarrollo de la depresión [17].

Estudios recientes muestran alteraciones en los niveles de expresión del GLT-1 en pacientes con trastornos depresivos y en modelos animales de depresión. Zink et al., 2010, utilizando un modelo animal de depresión mostraron una suprimida expresión de EAAT2 en el hipocampo y la corteza cerebral [190].

Un estudio clínico indicó que los pacientes con trastornos de depresión mayor se acompañaron con un aumento del estrés oxidativo [193].

Múltiples estudios han demostrado cambios en el número o características de células gliales en cerebros adultos de pacientes con trastornos psiquiátricos o en modelos de ratón, incluyendo reducciones en los niveles de GFAP en áreas corticales y corticolímbicas prefrontales en un modelo de depresión en ratas [69].

1. Mecanismos Antidepresivos y Ansiolíticos

Aproximadamente un 30 a 40% de los pacientes con trastorno depresivo mayor tienen depresión resistente al tratamiento, que no responde a las terapias antidepresivas actualmente disponibles. Por lo anterior, es muy importante identificar mecanismos subyacentes a la depresión para desarrollar estrategias terapéuticas efectivas [113].

Los antidepresivos, inhibidores de la absorción de monoaminas y los inhibidores de la MAO-A, restauran el árbol dendrítico atrofiado de las neuronas del hipocampo en animales sometidos a CMS. Este efecto está asociado con una recuperación de la función sináptica y una restauración del volumen del hipocampo [165, 198].

La fluoxetina (FLX), un inhibidor selectivo de la recaptación de serotonina (5-HT), es uno de los antidepresivos más utilizados. Un estudio reciente demostró que el tratamiento con FLX inducía la expresión de EAAT2 (GLT-1) en el hipocampo y la corteza de rata normales [190].

La administración crónica de corticosterona, que resulta en un fenotipo depresivo, reduce la densidad de la espina en CA1 y tanto la columna neuronal como los déficits conductuales se recuperan con la administración crónica de fluoxetina [19].

Algunos efectos conductuales de los antidepresivos farmacológicos se bloquean mediante la abolición de la neurogénesis del DG en algunas especies [109].

En la región del DG del hipocampo, la generación continua de neuronas nuevas desempeña un papel crítico en la formación de los comportamientos cognitivos y

emocionales de los animales. El aumento de la producción de neuronas en la zona subgranular (SGZ), uno de los principales nichos neurogénicos en el cerebro, puede amortiguar el síntoma depresivo inducido por el estrés, cobrando vital importancia la neurogénesis en los efectos antidepresivos [193, 199].

La neurogénesis también se observa dentro del efecto terapéutico de varios fármacos antidepresivos.

Si bien, aún no está claro si la reducción de la neurogénesis en el hipocampo ocurre en personas deprimidas, se ha demostrado que cuando la neurogénesis hipocámpica se ablacionó en ratas, las respuestas conductuales al tratamiento antidepresivo crónico se bloquearon, sugiriendo un papel causal para la neurogénesis en la eficacia de los antidepresivos [14].

Uno de los hallazgos más consistentes de los estudios post mortem es que los pacientes deprimidos muestran una disminución del tejido glial. Esto incluye una disminución del número de astrocitos y oligodendrocitos en la corteza prefrontal [178].

Estudios demuestran que los niveles de BDNF disminuyen en la sangre de pacientes deprimidos y sus niveles aumentan con el tratamiento antidepresivo [145].

El hallazgo de que los niveles séricos de BDNF se reducen en pacientes diagnosticados con MDD implica un posible papel del BDNF en la fisiopatología de la depresión. Además, un experimento de knock-out de BDNF en la circunvolución dentada dorsal del hipocampo fue capaz de inducir un comportamiento depresivo en ratas, sugiriendo que la producción reducida de BDNF y, por ende, la neuroplasticidad, pueden conducir a la depresión [15].

Diversos investigadores postulan que el déficit de BDNF en el hipocampo juega un papel esencial en la fisiopatología de la depresión, y la restauración de esta neurotrofina puede representar un mecanismo antidepresivo muy importante [157]. En otro estudio, el BDNF reducido asociado con el estrés por restricción se evitó mediante tratamiento con antidepresivos [182, 196].

Se ha demostrado que la fluoxetina aumenta la expresión de ARNm de BDNF en la circunvolución dentada del hipocampo, el área tegmental ventral (VTA) y el núcleo accumbens [15].

Se informan niveles disminuidos de mTORC en cerebros postmortem de individuos con trastornos del estado de ánimo relacionados con el estrés, mientras que la ketamina antidepresiva de acción rápida aumenta la señalización de mTORC en PFC de rata [137].

2. Hipótesis neurotrófica

La hipótesis neurotrófica de la depresión postula que los niveles bajos de BDNF conducen a alteraciones funcionales y estructurales específicas y, en última instancia, inducen comportamientos relacionados a la depresión. Hipótesis que se basa en que, en el hipocampo, los antidepresivos estimulan la neurogénesis y aumentan el número de conexiones sinápticas, a través de la vía AMPc, PKA, CREB, BDNF, trkB [17]. Estos aumentos experimentales en los niveles del BDNF hipocampal producen efectos similares a los antidepresivos [200].

La hipótesis neurotrófica descrita anteriormente es consistente con la observación de que ciertas subpoblaciones de pacientes deprimidos muestran pequeñas reducciones en el volumen total del hipocampo con el consiguiente aumento ventricular [201].

Además, la sobreexpresión de BDNF específicamente en los astrocitos del hipocampo produce efectos ansiolíticos, similares a los antidepresivos [18].

3. Ácidos Grasos Omega-3 en Depresión

Un metanálisis publicado recientemente indicó que un patrón dietético saludable, caracterizado por una alta ingesta de frutas, verduras, pescado y granos integrales, se asoció significativamente con un menor riesgo de depresión [186].

En el meta-análisis efectuado por Li et al, 2015, con un total de 26 artículos incluidos, sobre la base de 150.278 participantes, pudieron concluir que un mayor consumo de pescado se asocia significativamente con un menor riesgo de depresión [202].

Muchos estudios han informado que los n-3 PUFA son efectivos para la prevención y mitigación de enfermedades mentales; sin embargo, su efectividad permanece poco clara [203].

Debido a que la depresión y la ansiedad comparten muchas características similares, también se ha investigado la posibilidad de que los n-3 PUFA tengan efectos ansiolíticos. Se ha demostrado que los niveles de n-3 PUFA intracelulares en pacientes que experimentan ansiedad y depresión son bajos y que la ansiedad puede aliviarse mediante la adición de PUFA de tipo n-3 [204].

I. ANSIEDAD

Durante estos últimos años se han levantado dos teorías sobre el desarrollo de trastornos del estado de ánimo [22]:

a) Una activación excesiva de las vías inflamatorias y,

b) Alteraciones en el metabolismo del glutamato.

La evidencia reciente indica que estas dos vías convergen a nivel de la glía para dar lugar a alteraciones del comportamiento en pacientes con trastornos del estado de ánimo.

Los trastornos de ansiedad son un importante factor de comorbilidad con la depresión y están asociados con una variedad de problemas de salud, que pueden conducir a la disminución general de la calidad de vida experimentada por individuos ansiosos. Además, los trastornos de ansiedad representan una carga económica importante porque perjudican el rendimiento en el lugar de trabajo. Como tal, los trastornos de ansiedad son devastadores en sus costos personales, sociales y financieros [136].

Los estudios de pacientes con trastornos de ansiedad han mostrado niveles elevados de peroxidación lipídica en el trastorno de ansiedad generalizado y actividad antioxidante reprimida en el trastorno de pánico [205].

Muchas zonas cerebrales (hipocampo, amígdala, corteza prefrontal) han sido asociadas con los neurocircuitos de la ansiedad en humanos [136].

Una consecuencia de la exposición al estrés en roedores es una mayor ansiedad.

J. PTSD

Los trastornos relacionados con el trauma y el estrés, incluido el trastorno de estrés postraumático (posttraumatic stress disorder, PTSD), se encuentran entre los trastornos neuropsiquiátricos más prevalentes y debilitantes en el mundo.

El PTSD es una afección grave y con frecuencia discapacitante, que afecta aproximadamente al 8% de la población general, en algún momento de su vida. Entre estos pacientes, las comorbilidades psiquiátricas y/o médicas son comunes; muchas de ellas presentan una enfermedad de aparición temprana, enfermedad cardiometabólica, trastornos neurocognitivos y demencia [205].

El PTSD es un trastorno mental relacionado con el estrés que se desarrolla después de un evento extremadamente estresante, como por ejemplo la violencia física o el combate militar.

El DSM-V divide los síntomas del PTSD en cuatro categorías básicas [141]:

a) Síntomas intrusivos: se refiere a pesadillas, recuerdos y recuerdos repentinos e incómodos de eventos relevantes para el trauma. Es importante destacar que estos recuerdos pueden involucrar uno o todos los cinco sentidos, los olores a menudo son los más inquietantes, tal vez porque el sentido del olfato está menos sujeto a la modulación de PFC. Los flashbacks pueden ser tan vívidos que las personas, afligidas, pueden recrear el trauma.

b) Síntomas de evitación: incluye todos los comportamientos que impliquen evitar pensamientos, sentimientos o recordatorios relacionados con el trauma, como personas, lugares o cosas.

c) Las alteraciones negativas en la cognición y el estado de ánimo: es una categoría que incluye puntos de vista distorsionados y negativos de uno mismo y de los demás. Puede haber un interés disminuido en las actividades diarias y una alienación de los demás, incluso de los seres queridos. El afecto y las emociones pueden estar cada vez más limitados a eventos relevantes para el trauma, como la ira, la culpa o la vergüenza.

d) Alteraciones en la excitación y la reactividad: es la cuarta categoría amplia. Además de los signos de hiperexcitación e hipervigilancia, existe mayor irritabilidad y/o agresividad, imprudencia y concentración alterada; todas las cuales se asocian con disfunción de la corteza prefrontal.

La corteza prefrontal proporciona una regulación del comportamiento, pensamiento y emoción, generando las representaciones mentales necesarias para un

comportamiento flexible, incluida la capacidad de inhibir los impulsos inapropiados, la regulación de la atención, la percepción sobre las propias acciones y las de los demás [141]. Por lo tanto, puede proporcionar una dirección coordinada de la fisiología cerebral para una respuesta calmada, racional y flexible.

Estudios mediante resonancia magnética ha revelado la correlación entre el PTSD y un menor volumen en la zona CA3 (atrofia neuronas piramidales) y giro dentado (atrofia astrocitos) del hipocampo en humanos, que se condice con lo encontrado en modelos animales [135].

En relación a los síntomas del PTSD, las lesiones de la PFC deterioran la capacidad de concentrar o enfocar la atención y pueden debilitar el control de los impulsos y producir un comportamiento imprudente [141, 206].

Los trastornos de estrés, como el trastorno de estrés postraumático, a menudo se acompañan de reducciones volumétricas del hipocampo (también reportado en el desorden depresivo recurrente), de las cortezas límbicas asociadas y también de la corteza prefrontal [167].

La investigación sobre el desarrollo de tratamientos antiinflamatorios para el PTSD, hasta la fecha, se ha limitado a modelos animales. Por ejemplo, la administración de ibuprofeno a ratas sometidas a un estresor sustancial mostró una disminución del comportamiento ansioso y una expresión reducida de marcadores inflamatorios en el hipocampo, en comparación con las ratas a las que no se administró el medicamento [205].

La prueba de condicionamiento de miedo contextual es un paradigma bien establecido para investigar los mecanismos neuronales del aprendizaje y la memoria dependiente del hipocampo en roedores [78, 142].

K. NEUROINFLAMACIÓN

La inflamación es una respuesta innata beneficiosa ante la lesión tisular; sin embargo, una respuesta inflamatoria crónica o demasiado fuerte puede contribuir significativamente al daño tisular. Se cree que la inflamación excesiva en el cerebro exacerba las lesiones agudas, incluido el accidente cerebrovascular, y las enfermedades crónicas, como la esclerosis múltiple y la enfermedad de Alzheimer [207].

La interacción entre la inflamación y la respuesta al estrés es bidireccional, con inflamación que provoca la regulación al alza del eje HPA, donde los glucocorticoides y el sistema nervioso simpático aumentan también la inflamación [15].

Mucha evidencia actual de la literatura muestra los efectos perjudiciales de las citoquinas proinflamatorias (citoquinas clásicas como la IL-1β, IL-6, TNF-α) sobre la neurogénesis y la cognición del hipocampo.

La intervención terapéutica para trastornos cognitivos que se dirigen tanto a los mediadores inflamatorios como a la neurogenética puede ser un foco importante de investigación [14].

El estudio de la neuroinflamación como un importante contribuyente a la neurodegeneración, data desde la demostración de que la microglia alterada produce un fenotipo neurodegenerativo en los seres humanos [208].

La neuroinflamación crónica es una característica patológica común en el envejecimiento normal, así como en afecciones neurodegenerativas y se ha demostrado que afecta negativamente la neurogénesis hipocampal y los procesos cognitivos a lo largo de la vida. Por el contrario, los moduladores positivos de la neurogénesis del hipocampo adulto y la función cognitiva asociada, incluyen el enriquecimiento ambiental, el aprendizaje y el ejercicio [14, 209].

La inflamación asociada con un aumento de la IL-1, reduce la proliferación celular y la supervivencia de nuevas neuronas en el giro dentado de la rata adulta. La administración de IL-1β en sí misma disminuye la proliferación celular y la diferenciación de nuevas neuronas en el giro dentado del ratón adulto [30].

Un metanálisis reciente, que evaluó 24 estudios de citoquinas, con pacientes que cumplían los criterios del DSM para la depresión, reveló niveles elevados de IL-6 y TNF-α; sin embargo, los niveles de IL-1β, IL-2, IL-4, IL-8, IL- 10, e IFN-γ no se vieron afectados significativamente [210]. Un segundo metanálisis informó además que IL-1β, IL-6 y el marcador inflamatorio, proteína C-reactiva, también se asociaron positivamente con la depresión [196].

Se ha demostrado que ciertas citoquinas inducen un comportamiento similar a la depresión en roedores y primates. Un ejemplo de esto es la IL-1β, en el hipocampo, donde juega un rol importante en la mediación de los efectos anhedónicos y antineurogénicos del estrés crónico, a través del factor de transcripción NFκB (factor nuclear-κβ) [201].

NFκB es un factor de transcripción crucial en cualquier reacción inflamatoria. La activación de NFκB y la transcripción dependiente de NFκB de los factores proinflamatorios, son relevantes para la amplificación de los procesos inflamatorios y neurodegenerativos [100].

Los pacientes deprimidos muestran citoquinas proinflamatorias elevadas, como el TNF y la IL-1β, que se revierten con el tratamiento antidepresivo [137].

La administración de IL-1β o TNF-α, típicamente engendra una serie de síntomas conductuales (efectos soporíferos, ptosis, anorexia, fiebre, fatiga, actividad motora reducida, postura corporal rizada) conocidos como "comportamientos de enfermedad". De forma similar, estas mismas citoquinas también tienen potentes efectos activadores sobre el eje HPA [196].

Se ha demostrado que las elevaciones en la IL-1β en el hipocampo bloquea completamente la LTP en el hipocampo, in vitro e in vivo. Siendo LTP uno de los principales mecanismos celulares subyacentes al aprendizaje y la memoria [95, 211, 212].

Se ha demostrado que las citoquinas y los glucocorticoides disminuyen las monoaminas y reducen la neurogénesis. Un hallazgo importante es que un modelo de estrés crónico impredecible ha demostrado que disminuye los niveles de monoaminas en ratas [15].

Del mismo modo, la estimulación inflamatoria crónica de los astrocitos reduce la capacidad glial para generar y liberar mediadores neurotróficos, como el factor neurotrófico derivado de las células gliales (GDNF) para el soporte de las neuronas espectadoras [37].

Un posible mecanismo a través del cual la inflamación puede llevar a la depresión radica en la relación entre la inflamación y la neuroplasticidad, a través de mecanismos como la poda sináptica. Es posible que el aumento de la inflamación desempeñe un papel en la depresión de una manera similar, a través de la reducción de la complejidad dendrítica, que conduce a una conectividad funcional reducida en los circuitos de procesamiento de afectos [15].

Las alteraciones de la memoria inducidas por citoquinas proinflamatorias pueden implicar la regulación a la baja del BDNF [95].

El estrés oxidativo y la apoptosis están aparentemente vinculados en la fisiopatología de muchas enfermedades neurodegenerativas [143].

Se cree que la activación microglial contribuye a la fisiopatología de PD, mediante la liberación de factores proinflamatorios y neurotóxicos, como el factor de necrosis tumoral α (TNF-α), la interleucina 1β (IL-1β), IL-2, IL-4, IL- 6, interferón-γ, quimiocinas y especies reactivas de nitrógeno (por ejemplo, óxido nítrico [NO] y peroxinitrito), que desencadenan o exacerban la neurodegeneración en la PD [213].

Las alteraciones del metabolismo energético del cerebro y el estrés oxidativo son características de los trastornos neurodegenerativos, especialmente en AD [214].

Actualmente se ha identificado a la neuroinflamación como un tercer componente patológico de la enfermedad de Alzheimer [37]. Muchos estudios muestran que la AD se caracteriza por un estado proinflamatorio crónico en el cerebro, que incluye astro y microgliosis y la activación de la cascada clásica del complemento [5].

En un estudio reciente, se demostró que IL-1α, TNF y C1q derivados de microglia inducían un subtipo de astrocitos reactivos, llamados astrocitos A1, que se cree contribuyen a la muerte neuronal en trastornos neurodegenerativos, como AD [215.]

El deterioro cognitivo con el envejecimiento se asocia con neuroinflamación y un aumento de las citoquinas proinflamatorias, incluida la IL-1b [207].

L. RECEPTOR NICOTÍNICO DE ACETILCOLINA (nAChR)

Los receptores nicotínicos de acetilcolina pertenecen a la superfamilia de canales dependientes de ligandos catiónicos cys-loop. Son activados por su agonista la acetilcolina (ACh) y diversos ligandos exógenos (nicotina, colina, entre otros) para ejercer su función moduladora sobre la excitabilidad y plasticidad sináptica [216].

Los nAChR se encuentran ampliamente distribuidos por todo el sistema nervioso central, por lo cual, es adecuado considerar que estén involucrados en la fisiopatología de muchas enfermedades y afecciones neurológicas, por ejemplo, la enfermedad de Alzheimer, la esquizofrenia y el autismo [217].

En especies de mamíferos, hay 16 subunidades nAChR (α 1-α 7, α 9, α 10, β1-β4, Ɣ, δ y ε) que pueden juntarse para generar diversos subtipos de nAChR. Además, algunas subunidades nAChR (como α7) forman nAChR homoméricas funcionales, que contienen cinco copias de la misma subunidad [218, 219].

A través de los años se han identificado una gran cantidad de ligandos selectivos de nAChR, que incluyen agonistas y antagonistas competitivos que se unen en el mismo sitio que la acetilcolina (sitio de unión ortostérico) [220].

Existen ligandos que pueden modular la función de nAChR, uniéndose a sitios que son distintos del sitio de unión de la acetilcolina (incluidos los sitios ubicados en el dominio transmembrana). Estos incluyen moduladores alostéricos positivos (PAM), moduladores alostéricos negativos (NAM), moduladores alostéricos silentes (SAM) y compuestos que son capaces de activar nAChR, a través de un sitio de unión alostérico (agonistas alostéricos) [220].

Los PAM no tienen ninguna actividad agonista por sí solos, pero cambian la capacidad del ligando ortostérico (agonista) para afectar la apertura del canal. Por lo tanto, los PAM pueden potencialmente aumentar la efectividad de la ACh (acetilcolina) endógena que se libera en la sinapsis y fortalecer el tono colinérgico sin activar directamente los nAChR [217].

La unión de ligandos a sitios alostéricos en nAChR puede dar como resultado la activación eficiente de nAChR en ausencia de agonistas ortostéricos.

Muchas revisiones han discutido los posibles usos terapéuticos de los moduladores alostéricos nAChR, por ejemplo, en el tratamiento de déficits cognitivos; depresión; dolor y cáncer [220, 221, 222, 223].

La activación de receptores nACh con nicotina previene la muerte de neuronas dopaminérgicas en un modelo de rata, de enfermedad de Parkinson. Además, se ha demostrado que la estimulación persistente de los receptores ACh previene la neurotoxicidad del glutamato potenciado por β-amiloide [224].

La nicotina, un potente agonista de los receptores nicotínicos de acetilcolina, puede tener efectos antiparkinsonianos [97].

Abundante evidencia experimental también sugiere que los nAChR están involucrados en procesos cognitivos como la atención, el aprendizaje y la memoria, en el procesamiento central del dolor, y en comportamientos psicológicos como la ansiedad y depresión [217, 225].

1. α7nAChR

Los α7nAChR se expresan abundantemente dentro del SNC, en particular en microglia y astrocitos [189].

El α7nAChR representa un subtipo nicotínico importante, altamente expresado en las regiones del cerebro de los mamíferos, incluyendo el bulbo olfatorio, la corteza cerebral, el hipocampo, el hipotálamo y la amígdala [8].

El subtipo α7nAChR se ha relacionado con déficits cognitivos y una variedad de trastornos y enfermedades neurológicas, como las enfermedades de Alzheimer y la esquizofrenia [216].

Importante señalar también que se han descubierto muchos PAM para receptores α7, y se han dividido comúnmente en dos categorías: (a) PAM de tipo I, que aumentan la respuesta del receptor evocada por agonistas y mantienen la cinética de respuesta (por ejemplo, desensibilización). (b) PAM de tipo II, que potencian la respuesta del receptor inducida por agonistas y reducen drásticamente la desensibilización [217, 218].

El subtipo α7 es algo atípico ya que tiene una sensibilidad relativamente baja a la acetilcolina, una alta permeabilidad al calcio y muestra una desensibilización muy rápida [220]. En cuanto a la función, la activación de α7 nAChRs da como resultado fuertes afluencias de calcio y sodio, que en el caso de una ubicación presináptica facilita la liberación de neurotransmisores [189, 226].

De los muchos subtipos posibles de nAChR que se han descrito previamente, los receptores α7 y α4β2 son los dos subtipos principales, ampliamente expresados en el cerebro, en particular en el hipocampo [217, 227, 228].

Se ha demostrado que la activación de α7nAChR con agonistas, o la potenciación con PAMs, mejora el aprendizaje y la memoria dependientes del hipocampo. Por el contrario, la inhibición de α7 nAChR en el hipocampo da como resultado importantes deficiencias de aprendizaje y de memoria [216].

El α7nAChR está implicado en las funciones cognitivas del sistema nervioso central. La modulación de α7nAChR se considera una opción muy importante para el tratamiento de trastornos cognitivos (demencia cognitiva, la esquizofrenia, enfermedad de Parkinson), la inflamación y la sepsis [229].

El α7nAChR humano se ha identificado como un objetivo potencial para el descubrimiento de fármacos terapéuticos y se ha implicado en una serie de trastornos neurológicos y psiquiátricos. Donde la modulación alostérica puede facilitar la activación de α7nAChR sólo con bajos niveles de desensibilización [218].

Por otro lado, la activación de nAChR protege a las células neurogliales, como los astrocitos, contra el estrés oxidativo, la apoptosis y la neuroinflamación. Además, estos receptores aparecen en las primeras células gliales e indirectamente regulan muchos eventos neuronales, como la liberación del transmisor y la plasticidad sináptica [38].

La apertura de α7 que contiene canales nAChR puede causar un aumento significativo en la concentración de Ca^{2+} intracelular, debido a la liberación de calcio mediado por un segundo mensajero de las reservas intracelulares o de canales de Ca^{2+} dependientes de voltaje [8].

Numerosos otros estudios demostraron que la subunidad α7 participa en nAChR presinápticos que facilitan la liberación de glutamato en varias sinapsis, y en el desarrollo de neuronas del hipocampo [8].

Se ha demostrado que α7nAChR contribuyen a la organización de las dendritas de neuronas individuales y modulan el ritmo del desarrollo fisiológico y morfológico de las neuronas adultas [230].

Existe evidencia que muestra que la disfunción de nAChR glial podría contribuir a la disrupción de la neurotransmisión en AD [38].

Con respecto a los astrocitos, la activación de α7nAChR es capaz de inhibir la liberación de TNFα y MAPK en los astrocitos activados por el ion 1-metil-4-fenilpiridinio (MPP^+). Además, α7 nAChR es protector para los astrocitos ya que, a través de la estimulación de α7 nAChR, la nicotina suprime la apoptosis de astrocitos inducida por el estrés oxidativo [231].

Los agonistas y antagonistas de α7nAChR son compuestos farmacológicamente relevantes adecuados para el tratamiento de múltiples disfunciones cognitivas y / o enfermedades asociadas a la inflamación [229].

Los α7nAChR tienen un rol muy importante en la función glial, donde ejecutan diversas acciones reguladoras y excitadoras. Además, estos receptores en las

células gliales, son importantes en la patogénesis y el tratamiento de la AD [38, 232, 233].

Varios fármacos dirigidos a α7nAChR demostraron su potencial en el tratamiento de pacientes esquizofrénicos [216].

En el estudio de Liu et al, 2015, demostraron que la nicotina inhibía la apoptosis de astrocitos inducida por H_2O_2, y este efecto antiapoptótico de la nicotina podría ser suprimido por MLA que es un antagonista selectivo α7nAChR, lo que implica que α7nAChR juega un papel crítico en la apoptosis de astrocitos [97].

En la periferia, la acetilcolina mediante la activación de los α7nAChR inhibe la síntesis de citoquinas, mientras que en el cerebro (y en particular el hipocampo), la nicotina o acetilcolina, mediante la activación de los α7nAChR, bloquea la liberación de toxinas proinflamatorias [234].

M. ACIDOS GRASOS POLIINSATURADOS DE CADENA LARGA

Los lípidos son componentes vitales de las membranas neuronales e influyen en la función cerebral en muchos aspectos. El colesterol y los ácidos grasos insaturados se enriquecen en la membrana sináptica, en la cual son determinantes importantes para una variedad de procesos bioquímicos [235].

El tejido cerebral está conformado predominantemente por lípidos que en su composición poseen ácidos grasos saturados, monoinsaturados y poliinsaturados. Y el cerebro, específicamente, está altamente enriquecido con ARA y DHA [236].

Hay dos familias principales de ácidos grasos poliinsaturados (PUFA), los n-6 y n-3 (también conocidos como omega 6 y omega 3). Los ácidos grasos poliinsaturados generalmente se consideran ácidos grasos esenciales, necesarios para mantener una fisiología normal, pero no pueden ser producidos por los mamíferos y deben ser proporcionados por la dieta.

EPA y DHA son abundantes en pescados grasos (18.7% EPA más DHA en salmón, 32.9% EPA más DHA en atún) [236].

En referencia a los n-6 PUFA tenemos al ácido linoleico (LA; 18:2n6) quien es el precursor de ácido araquidónico (ARA; 20:4n6), de cadena corta, esencial en la dieta [236].

El principal ácido graso omega-3 encontrado en el cerebro es el ácido docosahexaenoico (DHA), que comprende entre un 10 a 20% del contenido total de ácidos grasos, específicamente en las membranas celulares; mientras que los otros ácidos grasos omega-3 como ácido alfa linolénico (ALA), ácido eicosapentaenoico (EPA) y ácido docosapentaenoico (DPA) comprenden menos del 1% del total de ácidos grasos del cerebro [237, 238].

El metabolismo rápido por β-oxidación parece ser un mecanismo por el cual EPA y ALA se mantienen a concentraciones 200-500 veces más bajas que DHA, pero lo que no está claro cuál es la razón funcional de sus bajos niveles en el cerebro [207].

El cerebro de roedores contiene 36%-46% de ácidos grasos saturados, 18%-33% de ácidos grasos monoinsaturados y 18%-28% de PUFA [239]. Donde el DHA está más concentrado en la corteza frontal y el hipocampo (16-22% de los ácidos grasos totales) [240].

La evidencia muestra que el DHA, en modelos animales, es antiapoptótico, neurotrófico e importante para la plasticidad sináptica [207].

Se acepta ampliamente que los ácidos grasos de cadena larga poliinsaturados n-3 son cruciales para el crecimiento y desarrollo del cerebro infantil durante el embarazo y después del nacimiento [241].

Nuestro organismo necesita estos ácidos grasos para variados procesos fisiológicos, como la promoción del crecimiento y desarrollo celular, el mantenimiento de un sistema inmunitario saludable, la regulación de reacciones metabólicas y diversas vías de señalización celular.

Se ha demostrado que los ácidos grasos omega-3 poseen propiedades antiinflamatorias, antitrombóticas, antiarrítmicas y antiangiogénicas, por lo que se utilizan como posibles objetivos para el tratamiento/prevención de la diabetes, el accidente cerebrovascular, la enfermedad de Alzheimer y diversos trastornos cardiovasculares (como aterosclerosis, hipertensión, enfermedad coronaria) [242].

Varias publicaciones han informado que los aceites de pescado reducen la gravedad de enfermedades como: la diabetes, el SIDA, la caquexia por cáncer, la insuficiencia cardíaca crónica, sepsis y son una posible atenuante de la atrofia del músculo esquelético [243].

Los DHA actúan como precursores de algunos mediadores que regulan diversos procesos dentro del cerebro, como la neurotransmisión, la inflamación, la reacción inmune y la supervivencia neuronal. Los informes han sugerido que los ácidos grasos omega-3 influyen en el funcionamiento del cerebro, a través de factores neurotróficos y también se sabe que tienen efectos positivos sobre la cognición [244].

Los efectos metabólicos bien reconocidos de EPA y DHA incluyen la disminución de los niveles de triglicéridos (TG), colesterol y de lipoproteínas de muy baja densidad (LDL) [245].

La suplementación a largo plazo con EPA y DHA disminuye la ansiedad en modelos animales de adicción a las drogas. En humanos, los PUFA tienen efectos positivos en la fisiopatología de una amplia gama de trastornos relacionados con el estrés [146].

La suplementación con ω-3 puede aumentar los niveles de serotonina en el cerebro de ratas [246].

Los estudios en animales han demostrado que la producción de eicosanoides derivados de ácido araquidónico (el ARA, el sustrato principal para la síntesis de eicosanoides) como prostaglandina E2 (PGE2) se ve disminuida por la alimentación con EPA o DHA [247].

Un estudio publicado concluye que una ingesta de EPA de 1,35 gramos al día, durante 3 meses, no fue suficiente para influir en la producción de PGE2, en cultivo de células mononucleares estimuladas por endotoxina; mientras que una ingesta de EPA de 2.7 gramos al día disminuyó significativamente la producción de PGE2 [248], sugiriendo un umbral para un efecto antiinflamatorio de la EPA de entre 1,35 y 2,7 gramos de EPA por día.

ARA y DHA se acumulan durante el desarrollo del cerebro, especialmente durante el período perinatal: en humanos entre el comienzo del tercer trimestre y 2 años de edad y en roedores entre el 7 y el 21 día posnatal. Estos periodos corresponden a la maduración neuronal rápida, sinaptogénesis, y la expansión de la sustancia gris [236].

Evidencia disponible hasta ahora sugiere que los niveles de n-3 PUFA en el cerebro modulan la reactividad y la sensibilidad al estrés [146]; y varios trabajos han mostrado, también, que la deficiencia dietética de n-3 induce cambios en los niveles sinápticos de serotonina (5-HT).

La suplementación con omega-3 puede aumentar el nivel de las proteínas que previenen la atrofia dendrítica en el núcleo geniculado medial y colículo inferior de las ratas estresadas. Por otro lado, los efectos positivos de los ácidos grasos omega-3 en el aprendizaje pueden haber sido por un efecto directo en la amígdala lateral, una zona del cerebro clave para el aprendizaje del miedo [249].

Por último, fuera del cerebro, los n-3 PUFA median la protección cardiovascular, previenen la pérdida ósea en la osteoporosis, y en términos generales, son efectores positivos en diferentes tipos de procesos inflamatorios, mejorando, por ejemplo, los resultados en la artritis reumatoide [250].

1. Efecto Estructural y Neurocognitivo de los n-3 PUFA

El DHA aumenta significativamente la densidad de las espinas dendríticas en las células piramidales del hipocampo y en las células de Purkinje del cerebelo [251].

El DHA afecta la regulación del factor de crecimiento que puede ser responsable del aumento del crecimiento de neuritas y la formación de sinapsis.

Los PUFA regulan la migración celular y la apoptosis, contribuyen a la sinaptogénesis y se incorporan a la transmisión sináptica colinérgica, catecolaminérgica y serotoninérgica.

El DHA protege a los astrocitos, la oligodendroglia y las células ganglionares de la retina, del estrés oxidativo y elimina los compuestos intracelulares libres inducidos por anión superóxido, peróxido de hidrógeno y radical hidroxilo [251, 252].

La EPA también puede ejercer un mayor efecto neurotrófico en comparación con el DHA, y se ha demostrado que los suplementos de EPA aumentan los niveles del BDNF después de una lesión cerebral traumática [200].

Los PUFA, muy abundantes en las membranas neuronales, previenen la apoptosis e influyen en componentes específicos de la señalización de supervivencia, como la fosfoinositol 3-quinasa (PI3K) [251].

Estudios en roedores han concluido que niveles bajos de DHA en el cerebro conducen a un pésimo rendimiento en una variedad de tareas cognitivas y de aprendizaje [253].

Actualmente son muchas las investigaciones referentes a la asociación entre la dieta rica en PUFA n-3 y los desórdenes del estado de ánimo. Donde los pacientes que sufren trastornos del estado de ánimo muestran niveles reducidos de EPA y DHA en regiones cerebrales específicas [236].

Sobre el efecto de los n-3 PUFA en el comportamiento emocional, los trabajos proponen diversos mecanismos como el aumento de la proliferación neuronal, aumento de las concentraciones de monoaminas en el cerebro e influencia sobre los niveles del BDNF [203].

Los efectos de los PUFA n-3 en los síntomas similares a la ansiedad se han relacionado con la acción del factor neurotrófico derivado del cerebro (BDNF), que era uno de los principales objetivos regulados por los n-3 PUFA [254].

La ingesta de alimentos ricos en n-3 PUFA se asocia con una menor prevalencia de depresión mayor, depresión posparto o trastorno bipolar.

Considerando algunas investigaciones, existen diferencias biológicas sustanciales entre el DHA y el EPA, quien posee un mayor efecto antiinflamatorio en el cerebro que el DHA y que el ácido α-linolénico, lo que puede contribuir a su supuesto mayor efecto antidepresivo [200].

El efecto ansiolítico de los suplementos de ω-3 puede estar relacionado con el aumento de los niveles de serotonina en el cerebro de ratas con estrés crónico. La serotonina tiene un papel clave en la regulación de comportamientos similares a la ansiedad [146].

Las concentraciones de EPA/DHA utilizadas en el estudio de Oshima et al. (2017), similar a la dieta enriquecida con EPA utilizada en el estudio Song et al., (20% de EPA y 8% de DHA), demuestran claramente un significativo efecto ansiolítico de EPA por sobre DHA [203].

Dos estudios prospectivos independientes han demostrado que los niveles más bajos de DHA en plasma están asociados con un mayor riesgo de desarrollar AD más adelante en la vida [255].

Estudios en humanos han demostrado que la suplementación alimenticia con ácidos grasos ω-3 mejora la función cognitiva y puede reducir el riesgo de desarrollar enfermedad de Alzheimer [256].

2. Efecto Antiinflamatorio de los n-3 PUFA

Muchas investigaciones han demostrado que DHA y EPA ejercen efectos protectores (actividad antiinflamatoria y antiapoptótica), en diferentes modelos experimentales e incrementan la síntesis del BDNF y la actividad antioxidante [237].

Los fosfolípidos de las células sanguíneas, involucradas en los procesos inflamatorios, en humanos que consumen una dieta occidental típica contienen aproximadamente 15 a 20% de ácidos grasos como ARA, 0.5 a 1% como EPA y 2 a 3% como DHA. El aumento de la ingesta de ácidos grasos ω-3 marinos da como resultado cantidades mayores de EPA y DHA (y también DPA) en estos fosfolípidos [247].

En humanos, un mayor consumo de n-3 PUFA se asocia con un menor riesgo de trastornos neurológicos asociados a la inflamación. Varios estudios epidemiológicos y observacionales informan que los sujetos con niveles más altos de n-3 PUFA en sangre tienen menor producción de citoquina proinflamatoria [236].

En un estudio de intervención prospectivo y doble ciego, se descubrió que los ácidos grasos poliinsaturados de cadena larga mejoraron las funciones ejecutivas y aumentan el volumen de materia gris, así como la microestructura de la sustancia blanca en individuos sanos mayores, después de 26 semanas de suplementación con aceite de pescado [241].

Los n-3 PUFA regulan negativamente la expresión génica inflamatoria, como también la de citoquinas o enzimas implicadas en la síntesis de eicosanoides, al tiempo que inducen mediadores lipídicos implicados en la resolución de la inflamación [257].

Las concentraciones de n-3 PUFA se asociaron con niveles más bajos de marcadores proinflamatorios (IL-6, TNFα, proteína C reactiva) y concentraciones más altas de marcadores antiinflamatorios (IL-10, factor de crecimiento TGF-β1). Por lo tanto, los autores concluyeron que los ácidos grasos n-3 son beneficiosos en pacientes afectados por enfermedades caracterizadas por inflamación activa [63].

Cuando se mantiene la producción de citoquinas proinflamatorias, estas moléculas se vuelven neurotóxicas, lo que provoca daño neuronal en muchas patologías cerebrales [236, 258].

Los efectos antiinflamatorios de los PUFA omega-3, se comprende desde el punto de vista que compiten con el ácido araquidónico (ARA), el principal representante de los n-6 PUFA. ARA es el precursor de prostaglandinas, tromboxanos y leucotrienos, que tienen efectos proinflamatorios. Por el contrario, los n-3 PUFA son precursores de otra clase de lípidos (protectinas y resolvinas) que tienen efectos antiinflamatorios [237].

Dado que la ingesta aumentada de ácidos grasos n-3 marinos disminuye la cantidad de ARA en los fosfolípidos de membrana de las células implicadas en la inflamación, podría esperarse que la producción de mediadores derivados de ARA se redujera simplemente debido a una cantidad reducida de sustrato disponible [247].

El potencial de la EPA para inhibir la conversión del ácido araquidónico en sus metabolitos proinflamatorios (principalmente prostaglandinas y leucotrienos) es el mecanismo molecular más aceptado detrás de la descripción de sus propiedades antiinflamatorias [242].

En cuanto a la neuroinflamación y DHA, se ha encontrado una disminución en la infiltración de leucocitos / neutrófilos, en la activación de NF-kB y en la expresión de COX-2 en animales tratados con DHA, mientras que otros estudios encontraron un aumento de GFAP en animales tratados con DHA [207].

Inoue et al, 2017, publicaron el primer estudio que demuestra los múltiples efectos antiinflamatorios de los ácidos grasos poliinsaturados n-3 EPA y DHA en la microglía activada. Ellos sugieren que estos PUFA pueden suprimir la neuroinflamación crónica asociada con la obesidad y la diabetes, al reducir la liberación de IL-6 y TNF-α de la microglia activada [259].

El DHA reduce marcadamente la fosforilación inducida por IL-1 de I-kβ (inhibidor del factor nuclear-kβ), evitando así su degradación, lo que posteriormente hace que NF-kβ se encuentre en estado inactivo. Por lo tanto, al inhibir la activación de NF-kβ y su subsecuente translocación al núcleo, el DHA afecta directamente su señalización aguas abajo e impide la activación de sus dianas génicas, que incluyen principalmente la producción de citoquinas (tales como TNF-α) [237].

La dieta rica en EPA atenúa la producción de la citoquina proinflamatoria IL-1β y mejora el deterioro de la plasticidad sináptica en el hipocampo de ratas viejas [236]. Algunos estudios, en animales, informan que los ácidos grasos ω-3 marinos, EPA, aumentan la concentración de la citoquina antiinflamatoria IL-10 [247]. Entonces, muy importante, el EPA regula negativamente la liberación de citoquinas inflamatorias que pueden producir síntomas clínicos de depresión, especialmente IL-1β, TNFα e IL-6 [200].

Mizunoya et al. comparó el efecto del aceite de pescado y la manteca de cerdo con la ansiedad e informó que el comportamiento similar a la ansiedad en la prueba de laberinto elevado (elevated pluz maze, EPM) se redujo en el grupo que consumió aceite de pescado [203].

El efecto de los n-3 PUFA tiende a ser especialmente robusto cuando los animales utilizados en los experimentos se encuentran en estado de estrés [203]. La importancia del DHA en la respuesta de los astrocitos a los glucocorticoides ayuda a comprender el papel de n-3 PUFA en la protección del cerebro contra el daño inducido por el estrés [158].

El DHA atenúa la respuesta de los astrocitos a la corticosterona, en parte, al disminuir los receptores de corticoides activados durante el estrés. Por su parte, la corticosterona favorece la incorporación de DHA en las membranas de los astrocitos, lo que sugiere un ciclo protector que favorece una función astroglial adecuada, en un cerebro sometido a estrés crónico.

En un reciente estudio realizado por Zgórzyńska et al, publicado el año 2017 (donde se incubaron células astrocíticas primarias de ratas con DHA y EPA), mostraron que n-3 PUFA mejora la defensa antioxidante en los astrocitos, a través del mecanismo dependiente de Nrf2. Estos resultados sugieren que el enriquecimiento de los astrocitos con N-3 PUFA puede proteger mejor a las neuronas durante condiciones perjudiciales [260].

N. KRILL OIL

En los últimos años, ha habido un aumento notable en la investigación sobre el aceite de krill (Krill Oil, KO) por sus beneficios para la salud [256].

El Krill Euphausia superba (krill), es un pequeño crustáceo marino (con un 12-50% de contenido lipídico) y una de las especies más importantes y abundantes de la zona marina antártica, comprendiendo alrededor del 50% de la biomasa del zooplancton [143, 261].

El KO contiene varios potentes antioxidantes como el carotenoide astaxantina (soluble en grasas) [256], y vitaminas A y E [245], y se caracteriza por una alta concentración de ácidos grasos n-3 PUFA de cadena larga en forma de fosfolípidos (PL) (30-65%) [262] y estos a la vez, mayoritariamente en forma de fosfatidilcolina (también llamada lecitina, uno de los principales constituyentes de las bicapas lipídicas de las membranas celulares) [256].

Los PL, en forma de fosfatidilcolina, comprenden alrededor del 40% del aceite de krill en base p/p. Se cree que esta unión de ω-3 a la fosfatidilcolina contribuye a los beneficios para la salud asociados con el aceite de krill [256].

Los estudios han demostrado n-3 PUFA de cadena larga en forma de PL, a diferencia de la forma triacilglicerol, es más biodisponible y se absorbe de manera muy eficiente por los tejidos del cerebro [254, 262].

El consumo humano de krill se ha sugerido cada vez más como una estrategia nutricional potencialmente saludable, especialmente con respecto a la mejora de la respuesta inmune, disminuyendo el riesgo de enfermedades cardiovasculares y proveyendo neuroprotección contra la pérdida cognitiva progresiva [143].

Los resultados del metanálisis realizado por Ursoniu et al, 2016, sobre la base de ensayos controlados aleatorizados, mostraron una reducción significativa en las concentraciones plasmáticas de colesterol LDL y TG y un aumento significativo en las concentraciones plasmáticas de colesterol HDL después de la suplementación con KO (por al menos 2 semanas), mientras que la reducción en los niveles plasmáticos de colesterol total no alcanzó significación estadística [245].

Deutsch (2007), informó que la mayoría de los importantes efectos antiinflamatorios obtenidos de la administración de KO a corto plazo (300 mg / día durante 7-14 días) en pacientes con osteoartritis se relacionó con la inhibición de la formación de leucotrienos [143].

El estudio de Hals et al, 2017, demostró que el tratamiento a largo plazo con fosfolípidos (con una preparación altamente purificada, de 50 a 450 mg de fosfolípidos/kg/día) ricos en ω-3 extraída de KO, alteró los perfiles de lípidos en

sangre (en primates dislipidémicos/diabéticos no humanos). Redujo el colesterol total, LDL -colesterol y triglicéridos, mientras que aumentó el colesterol HDL [261].

Los ácidos grasos ω-3 son menos inflamatorios que el ácido araquidónico y otros ácidos grasos ω-6 y ω-9. Cuando el ácido araquidónico está presente, se produce tromboxano A2. Cuando los ácidos grasos ω-3 son la forma predominante, se producen formas menos inflamatorias de leucotrienos y prostaglandinas [256]. Debido a lo anterior, se habla que los ácidos grasos ω-3 tienen una acción "antiinflamatoria".

En el trabajo realizado por Cheong et al, 2017, el aceite de krill demostró su capacidad de proteger contra el daño oxidativo y la peroxidación de lípidos en modelo de ratones envejecidos (D-galactosa). Tras la administración con aceite de krill, obtuvieron un aumento significativo en los niveles séricos de superóxido dismutasa (SOD) y glutatión peroxidasa (GSH-Px) [262].

Los resultados de Li et al, 2018, muestran que el KO tuvo efectos favorables en la mejora de la función cognitiva y el alivio de la ansiedad mediante la reducción del nivel de Aβ42 (beta-amiloide 42) en el hipocampo y la mejora del daño oxidativo en el cerebro. El KO se puede aplicar como complementos alimenticios y/o ingredientes funcionales con efectos neuroprotectores [254].

Algunos trabajos también han revelado que el KO podría mejorar la sensibilidad y secreción de la insulina en un modelo de conejos con obesidad y atenuar la inflamación en ratones alimentados con alto contenido de grasa [254].

1. Astaxantina

La astaxantina (astaxanthin, ATX), un pigmento carotenoide, liposoluble, es un antioxidante biológico que existe naturalmente en algas, peces, crustáceos y aves [263].

La astaxantina es de color anaranjado rojizo oscuro y que contribuye a la pigmentación rosada del krill. Como todos los animales, el krill no es capaz de producir ATX endógenamente. Varias formas de algas (que son parte de la dieta del Krill), han sido reconocidas como la fuente original de ATX [256].

Muchos trabajos científicos han dejado de manifiesto que los carotenoides en la dieta, y en particular la astaxantina, desempeñan un papel en la regulación de la respuesta inmune, el daño oxidativo y la inflamación en humanos y en modelos animales [143].

Se ha demostrado que la astaxantina tiene poderosas actividades antioxidantes, al atrapar y atenuar ROS y radicales libres (anión superóxido, peróxido de hidrógeno, etc.) e inhibir la peroxidación lipídica in vitro [263].

La astaxantina realiza varias funciones beneficiosas en humanos, por ejemplo: la inhibición de la oxidación de los PUFA en membranas, protección contra la fotooxidación con luz UV en células cutáneas, modulación de respuestas inflamatorias exacerbadas, control de procesos carcinogénicos, ralentización del envejecimiento y enfermedades relacionadas con el envejecimiento [143].

En estudios recientes se ha demostrado que los efectos antioxidantes y antiinflamatorios de los ATX son beneficiosos para el tratamiento de enfermedades del sistema nervioso central, sin causar efectos secundarios o toxicidad [262].

La astaxantina inhibe significativamente la actividad de NF-κβ, un efecto que se asocia con su mayor actividad antioxidante [143]. Con una capacidad antioxidante 100 veces mayor que la del alfa-tocoferol (es decir, la vitamina E) [264].

El tratamiento con ATX después de la hemorragia subaracnoidea redujo significativamente la actividad del NF-κβ y la expresión de citoquinas inflamatorias, lo que mejoró la disrupción de la barrera hematoencefálica, el edema cerebral, la degeneración neuronal y, por ende, la disfunción neurológica [262].

Zhang et al, en el año 2017, trabajando con un modelo de lesión por estiramiento de astrocitos in vitro, concluyó que la ATX previno la expresión de NKCC1 (una proteína de membrana intrínseca que facilita el cotransporte de iones cloruro, sodio y/o potasio) al limitar los mediadores proinflamatorios mediados por NF-κB. Destacando el papel antiinflamatorio de ATX después del trauma en astrocitos cultivados [262].

En el aceite de krill producido comercialmente, los niveles de astaxantina son de aproximadamente 100 ppm en base p / p [256].

O. COTININA

La cotinina, un compuesto nootrópico (suplementos que buscan mejorar la facultad cognitiva), es un agonista débil de los receptores nAChR [167].

La cotinina (1-metil-2-(3-piridinil)-2-pirrolidinona, $C_{10}H_{12}N_2O$), es el metabolito predominante de la nicotina y se ha demostrado que mejora la ansiedad y la pérdida de memoria en modelos animales. Es específicamente un neuroprotector, potenciador de la memoria y compuesto antiinflamatorio.

Preserva la densidad sináptica, mejora la memoria, disminuye la ansiedad, y disminuye el comportamiento depresivo en animales expuestos a estrés [265]. Diversos autores han informado sobre los efectos de la cotinina en la memoria de trabajo y el comportamiento depresivo en ratones sometidos a una restricción prolongada de la movilidad.

Recientemente se ha sugerido que la cotinina puede actuar como un modulador alostérico positivo (PAM) del α7nAChR.[167]

La cotinina actúa como agonista de los subtipos tanto de α4β2 como α3α6β2 nAChR en la región caudada que conduce a la liberación de dopamina. Se ha sugerido, además, que la cotinina puede dirigirse a otros receptores potenciales, a través de la activación de los sistemas serotoninérgicos (5-HT) y dopaminérgicos [266].

En un modelo animal, la administración sistémica a largo plazo de cotinina en dosis hasta diez veces más altas que las obtenidas fumando tabaco redujo la ansiedad y facilitó la extinción de una memoria de miedo contextual [267].

Además, la cotinina influye positivamente en el aprendizaje, la memoria, la atención, la función ejecutiva y el comportamiento impulsivo en varios modelos animales. Por otra parte, cotinina retrasa la progresión de la pérdida de memoria en un modelo de ratón de la enfermedad de Alzheimer [167].

Se ha demostrado que la administración de cotinina contribuye a inhibición de glucógeno sintasa quinasa 3 beta (GSK3β) mediante la estimulación de la vía Akt / GSK3β in vitro e in vivo [268, 269].

Grizzell et al, 2014, utilizando un tratamiento con cotinina obtuvieron como resultado, un aumento en la forma inactiva de GSK3β en ratones RS (restricción crónica del movimiento) y aumento significativo en la expresión de sinaptofisina en PFC de ratones RS, aunque dicho aumento no era detectable en el hipocampo de esos animales.

La inhibición de GSK3β por la cotinina puede estar mediado por múltiples factores, incluida la mejora de la 5-HT y / o neurotransmisión de la dopamina [167].

La cotinina estimula Akt e inhibe GSK3β en el hipocampo y la corteza prefrontal. Se cree que el aumento anormal de la actividad de GSK3β y la consiguiente hiperfosforilación de Tau es un paso crucial en la formación de ovillos neurofibrilares, muerte celular neuronal y declive cognitivo en la EA [265, 270].

Parte de los resultados, adjuntos en esta tesis, demuestran que el tratamiento con cotinina reduce el comportamiento de tipo depresivo, en condiciones crónicamente estresadas y no estresadas.

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V. RESULTADOS Y DISCUSIÓN

Se adjuntan a continuación, en el contexto de su presentación como artículos en las revistas científicas.

Artículo N°1

Role of neuroinflammation and sex hormones in war-related PTSD

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Role of neuroinflammation and sex hormones in war-related PTSD

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ABSTRACT

The susceptibility to develop posttraumatic stress disorder (PTSD) is greatly influenced by both innate and environmental risk factors. One of these factors is gender, with women showing higher incidence of trauma-related mental health disorders than their male counterparts. The evidence so far links these differences in susceptibility or resilience to trauma to the neuroprotective actions of sex hormones in reducing neuroinflammation after severe stress exposure. In this review, we discuss the impact of war-related trauma on the incidence of PTSD in civilian and military populations as well as differences associated to gender in the incidence and recovery from PTSD. In addition, the mutually influencing role of inflammation, genetic, and sex hormones in modulating the consequences derived from exposure to traumatic events are discussed in light of current evidence.

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Abbreviations: BDNF, brain-derived neurotrophic factor; CPS, cold pressor stress; CSR, combat stress reaction; CRF, corticotrophin releasing factor; DHEA-S, dehydroepiandrosterone sulphate; DHT, dihydrotestosterone; ERα, estrogen receptor alpha; GR, glucocorticoid receptor; HPA, hypothalamus-pituitary-adrenal; HPG, hypothalamus-pituitary-gonadal; IL, interleukin; MRI, magnetic resonance imaging; OEF, operation enduring freedom; OIF, operation Iraqi freedom; OND, and operation new dawn; PVN, paraventricular nucleus of the hypothalamus; PTSD, posttraumatic stress disorder; TNFα, tumor necrosis factor-alpha; SIRs, Spontaneous intrusive recollections.

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1. Introduction

1.1. Posttraumatic stress disorder

PTSD is defined as a reaction following the experience of an unusually traumatic event that involved the threat of death or serious injury to the person or to others and in which the person felt intense fear, horror, and helplessness (Shalev, 2009; Bryant et al., 2011). Stressor exposure initiates physiological and

psychological responses that prepare an organism to cope with this perturbation in homeostasis. Fear elicited by imminent danger of death, triggers in split-second the "fight-or-flight" response that consists in neuronal, endocrine, and behavioral responses, that are directed to avoid or confront dangerous situations.

These healthy physiological reactions prepare individuals to escape from an imminent danger, but extinguishes after the event. The two most common and conspicuous expressions of combat-induced psychological disorders are combat stress reaction (CSR) and post-traumatic stress disorder (PTSD) (Mareth and Brooker, 1985). The psychological consequences of a traumatic event are multiple including cognitive, somatic, and psychiatric dysfunctions (Bosson et al., 2011; Chiu et al., 2011).

PTSD was first brought to public attention in relation to war veterans (Susskind and Chandrasekaran, 1987), but it can result from a variety of traumatic incidents, such as mugging, rape, torture, being kidnapped or held captive, child abuse, car accidents, train wrecks, plane crashes, bombings, or natural disasters such as floods or earthquakes (Shalev, 2009). PTSD is characterized by seven symptom clusters, including re-experiencing-the traumatic events as vivid memories of the trauma (flashbacks), sleep disturbances (nightmares, sleeplessness); avoidance-numbing of responsiveness to, or reduced involvement with, the external world (Cohen et al., 2011; Peri et al., 2000); negative affect-dysphoria and negative appraisals about oneself and the world (Chiu et al., 2011); anhedonia; aggressive-externalizing behaviors; anxious and dysphoric arousal symptoms such as hyperarousal (Orr et al., 1995), hyper-vigilance, fear, anxiety, irritability, guilt/shame, dissociative symptoms, and as well as cognitive impairments (Shalev, 2009; O'Donnell et al., 2003; Guez et al., 2011; Orr et al., 2003).

The hypothalamus-pituitary-adrenal (HPA) axis has an important role in mediating stress responses in PTSD (Yehuda et al., 1991; Yehuda, 1998; Fenchel et al., 2015; Kasckow et al., 2001; Rohleder et al., 2010). Changes in glucocorticoid sensitivity in patients with PTSD have been also well documented (Rohleder et al., 2010). Evidence suggests that corticotrophin releasing factor (CRF) is elevated in brains of patients with depression and PTSD, perhaps as a result of CRF hypersecretion (Bauer et al., 2010; Gold et al., 1984; Gold and Chrousos, 1985, 2013). For example, patients with PTSD have higher levels of CRF in their cerebrospinal fluid, which are thought to reflect high levels of central CRF release (Kasckow et al., 2001; Bremner, 2003). Post-mortem studies have confirmed increased CRF expression in the brains of depressed patients (Banki et al., 1987) and increased expression of the CRF_1 receptor subtype, implicated in HPA axis and anxiety behavior (Hauger et al., 2009, 2006, 2012). Additionally, single nucleotide polymorphisms on the CRF_1 gene are found in patients with stress-related psychiatric disorders (Wasserman et al., 2008, 2009, 2010; Polanczyk et al., 2009). A recent report showed evidence that the CRF1 antagonist SSR125543 prevented sleep disturbances in a mouse model of PTSD (Philbert et al., 2015).

1.1.1. Posttraumatic stress disorder in war zones

1.1.1.1. Development of PTSD in soldiers and veterans of war. Participation in combat situations is a known stressor that can lead to PTSD. Soldiers are exposed to dangers, such a loss of life, injury, and the death of comrades, as well as to harsh physical conditions, such as extreme weather, lack of sleep, lack of food, isolation, loneliness, and loss of family support (Karstoft et al., 2013; Goldmann et al., 2012). PTSD is a major physical and mental health problem for military personnel, their families and civilians exposed to trauma (Calhoun et al., 2002; Solomon et al., 1992). In fact, the incidence of PTSD is higher in combat-exposed soldiers (10–40%) than in non-combat -exposed civilian population (around 6–7%).

Stressful experiences that occur during a traumatic event have consistently been implicated in the severity of reaction. In this regard, some studies have implicated both objective and subjective dimensions of trauma exposure in the development of future mental distress (Solomon et al., 1987; Minkowski, 2000), others have argued that one's subjective assessment of the event is the critical determinant of outcome (Solomon et al., 1987; Rosen et al., 2011; Ramchand et al., 2015; Solomon et al., 1988).

PTSD among veterans is about 3 times higher than the general population, but it may be 30 times higher in combat veteran depending on risk factors (Zohar et al., 2009; Richardson et al., 2010a), combat intensity (Boasso et al., 2015), and number of deployments (Marx et al., 2009). PTSD constitutes the most common psychiatric condition for which veterans seek services (Ramchand et al., 2015; Richardson et al., 2006). For example, 56% of Israeli soldiers who had presented stress-related pathological symptoms during the 1982 Lebanon war developed chronic PTSD (Solomon et al., 1989). In that same war, these pathological symptoms accounted for more than 20% of the total number of casualties. PTSD incidence is particularly high among those soldiers exposed to combat, with values ranging from 6% to 31% in US combat veterans (Richardson et al., 2010b; Dohrenwend et al., 2006). Behavioral therapy and drug treatment strategies have proven to be less effective in the veteran population with combat-PTSD than in the civilians. A reason for treatment failure in combat PTSD is a premature treatment dropout (12–39%). Considered altogether, an important percentage of individuals with PTSD do not respond to any type of treatment. Most patients subjected to cognitive processing therapy (49–70%) show considerable symptom improvement. However, two-thirds of these patients still score positive for PTSD after treatment (range, 60%–72%) (Steenkamp and Litz, 2014; Steenkamp et al., 2015).

In a different population, among UK military personnel who deployed to the 2003 Iraq war an incidence of PTSD between 3 and 6% was found (Hotopf et al., 2006). However, these numbers may be underestimated due to under-reporting of mental disorders in active duty personnel considering it a sign of weakness, loss of confidence, stigma, and a threat to their careers.

Studies have shown that war-related PTSD casualties report greater impairments in spousal intimacy, reduced sexual desire and more sexual dysfunction (Solomon and Dekel, 2008), as well as higher propensity to suffer from outbursts of rage and aggression and as a result, this population tends to report lower levels of family functioning (Solomon, 1988; Beckham, 1999; Kirby et al., 2012; Kirby and Yardley, 2008; Glenn et al., 2002; Beckham et al., 1997) and drug abuse (Calhoun et al., 2000).

1.1.1.2. Development of PTSD in civilians of war conflict zones. Also, citizens from countries in war conflicts or after terrorist attacks develop PTSD. The middle east has been afflicted by long-standing armed conflicts and wars. After Gaza conflict civilian in conflict zones have been diagnosed with PTSD (Thabet et al., 2004; Al-Krenawi et al., 2009; Palgi et al., 2012; Stein et al., 2013; Gil et al., 2015, 2016). Exposure to traumatic events can result in mental, health problems in children and adolescents. For example, Israeli and Palestinian citizens are at a disproportionately higher risk of experiencing PTSD than other comparable populations because of their prolonged exposure to political violence (Al-Krenawi et al., 2009; Schneider, 2007; Hamama-Raz et al., 2008). A recent review of the literature regarding mental health of children and adolescents living in Israel, Palestine, Lebanon and Iraq, explored prevalence and risk factors that influence the psychological consequences of exposure to armed conflict experiences. This analysis, including 71 studies selected from PubMed, revealed a prevalence of PTSD in children and adolescents living in these conflict zones of around 5–8% in Israel, 23–70% in Palestine and 10–30% in Iraq.

The risk factors associated to PTSD development were level and type of exposure, age, gender, socio-economic adversity, social support and religiosity (Al-Krenawi et al., 2009). Some studies have shown that Israeli citizens commonly report trauma- and stress-related mental health symptoms, the most frequent symptoms being avoidance/numbing (55.5% of the participants), followed by hyperarousal symptoms (49.4%), and re-experiencing trauma-related scenes (37.1%) (Bleich et al., 2003). On the other hand, the rate of post-traumatic stress reactions in Palestinian children who experienced war traumas is very high. A study including 239 children of 6–11 years of age show that of this sample, 98 (41%) reported moderate/severe PTSD reactions. The total number of trauma experiences was the best predictor of presence and severity of PTSD (Thabet and Vostanis, 1999). Another study examined among 139 adolescents between 12 and 17 years of age in Gaza the relationship between exposure to war stressors and psychological distress. The results revealed higher levels of PTSD symptoms in this population such as avoidance, depression, and intrusive symptoms, than similar communities not affected by war. Diagnosis of PTSD was 56.8% compared to 6.3% in peacetime populations. This study also revealed that significant risk factors for PTSD were exposure to violence, female gender, older age, and an unemployed father. Risk factors for anxiety were exposure, female gender, and older age whereas female gender was the only significant risk factor for depression (Krupnick, 2010). Overall, these and many other studies show a high incidence of PTSD in civilians living in war zones (Dubow et al., 2012a, 2010, 2012b; Qouta et al., 2003).

The urgent need to provide children and adolescents living in conflict areas with help make fundamental to understand the factors associated with the development of PTSD and the underlying mechanisms of resilience/susceptibility that may permit to provide these populations with needed therapies after and during conflict resolutions. The ability to cope with exposure to traumatic events seems to be greatly influenced by gender and age of the victims at the time of exposure, in which both factors are associated with hormonal differences.

1.1.2. Role of gender and sex hormones on the development of PTSD

1.1.2.1. Preclinical studies. It is broadly accepted that stress may trigger the onset and exacerbate the severity of several mental health and neurological disorders, including PTSD. Mood disorders have a higher incidence in women than men and is considered that resilience to stress is also modulated by several gender-associated factors. Changes in stress response linked to gender has been observed in animal models (Carvalho-Netto et al., 2011).

It has been well established that chronic stress produces morphological and functional changes in various regions of the brain. In a recent study in rodents, the authors examined sex-dependent differences in presynaptic innervation of the amygdala, bed nucleus of the stria terminalis (BST), paraventricular nucleus of the hypothalamus (PVN), and prefrontal cortex (PFC), in response to chronic stress (CS) in male and female rats. Following 14 days of stress, the expression of the presynaptic protein synaptophysin was examined. The results showed a higher expression of synaptophysin in females than males in all brain areas evaluated. After CS, the PVN, principal nucleus of the BST (BSTpr), and basolateral nucleus of the amygdala (BLA) displayed significantly reduced synaptophysin density in females but not males. Furthermore, males showed an increase in synaptophysin in the PVN after CS, suggesting a differential presynaptic modulation of PVN following CS. These data suggest marked sex differences in PVN, BSTpr, and BLA presynaptic innervation before and after CS likely associated with the different response to CS observed among males and females.

1.1.2.2. Clinical studies. Gender is also a factor in humans as women are considered according to many studies to be more affected by certain type of traumatic events (Bryant, 2003; Street et al., 2015; Nishi et al., 2015; Laufer and Solomon, 2009). Men and women present clear differences in susceptibility to disorders showing a marked immune component including PTSD. In fact, women have higher prevalence than men of many stress-associated conditions such as migraine (Cucurachi et al., 2006), insomnia (Singareddy et al., 2012) and irritable bowel syndrome (Cain et al., 2009). Also, current evidence suggests that females suffering from traumatic stress show higher levels of drug abuse, mental health problems and sex risk behaviors than stressed males and non-traumatized subjects (Guidetti et al., 2009; Stevens et al., 2003) (Table 1). Although men in average experience more traumatic events in life, trauma has been associated with more severe

Table 1
Gender differences for the development of posttraumatic stress disorder.

First author, year	Subject of study	Ref, sample size	Study characteristics	Findings
Bryant, 2003	Gender differences in the relationship between acute stress disorder and PTSD following motor vehicle accidents.	76,134 subjects	33 subjects with PTSD (36% male, 64% female)	More females were diagnosed with PTSD after experiencing motor vehicle accidents
Laufer and Solomon, 2009	Gender Differences in PTSD in Israeli Youth Exposed to Terror Attacks.	79, 2999 subjects	2136 subjects with PTSD (40% male, 60% female)	More PTSD symptoms in girls than in boys after terror attacks exposure
Stevens et al., 2003	Traumatic stress and gender differences in relationship to substance abuse, mental health, physical health, and HIV risk behavior in a sample of adolescents enrolled in drug treatment.	84, 378 subjects	274 male, 104 female adolescents	Females with acute levels of trauma symptoms had higher levels of substance use, greater HIV risk behaviors and mental and physical health problems, when compared to males and females with low levels of TS symptoms
De Bellis and Keshavan, 2003	Sex differences in brain maturation in maltreatment-related pediatric PTSD.	90, 61 subjects	Children and adolescents, with chronic PTSD (31 males, 30 females)	Subjects with PTSD did not show normal age-related changes in volume in several brain regions. This finding was more prominent in males than females.
Jacobson et al., 2015	Longitudinal assessment of gender differences in the development of PTSD among US military deployed personnel	150, 4684 subjects	2342 male, 2342 females	Findings suggest that women do not have a significantly different risk for developing PTSD than men after experiencing combat.
Rohleder et al., 2001	Sex differences in glucocorticoid sensitivity of proinflammatory cytokine production after psychosocial stress.	17, 45 subjects	27 males, 18 females	These results show similar cortisol responses to stress among men and women, but gender may exert differential effects on the immune system by modulating GC sensitivity of proinflammatory cytokine production.

psychiatric disorders among women (Rivera et al., 2015). However, some studies have suggested that gender can be a risk factor depending on the context of exposure to trauma. A study by Freedman et al. failed to find gender-related differences in recovery rates from PTSD in men and women that survived motor vehicle accidents (Freedman et al., 2002). Furthermore, Yasan et al. found no gender-related differences in PTSD prevalence among people living within an area of conflict, but reported the risk of PTSD for those who experienced military conflict was higher among men then woman. At difference, the gender difference in PTSD prevalence was about five times higher in women when assault was involved, but it decreased to three when sexual trauma was excluded from calculations (Stein et al., 2000).

Imaging analyses, focused on neuroanatomical aspects of PTSD revealed a pattern of stress-induced remodeling of brain regions, such as the prefrontal cortex and the hippocampus that participates in fear control in severely traumatized individuals (Shansky et al., 2009a).

Another recent MRI study of brains from abused children and youth with PTSD, showed lower cerebral volumes than controls (Bremner, 2002; De Bellis and Keshavan, 2003; De Bellis et al., 2002a; De Bellis et al., 2002b; Jackowski et al., 2009; Richert et al., 2006; Thomaes et al., 2012). These studies also revealed gender-related differences in the morphological changes induced by stress in the brain, with effects on brain volume more accentuated among males than females. Boys showed reduction of volumes of splenium and corpus callosum regions 1 (rostrum) and 6 (isthmus), but larger lateral ventricular volumes than controls and girls with PTSD (De Bellis and Keshavan, 2003) (Rohleder et al., 2001).

In the analysis of this data is important to consider that also under basal conditions, the brain areas that participate of the neuronal circuitry of fear, show gender-related differences in the size, synaptic connectivity and glial cell abundance (Palanza, 2001; Weinstock, 2007). These differences can be very pronounced, for example, in the anterior hypothalamus, the sexually dimorphic nucleus of the medial preoptic area, is 2.5–5 times larger in males than females (Gorski et al., 1978; Diamond et al., 1983; Fleming et al., 1986). Furthermore, the rostral anterior commissure, a fiber tract connecting the left and right hemispheres and several brain regions such as the amygdala, nucleus accumbens and piriform cortex, is bigger in females than in males (Jones et al., 1997). In addition, several sexual dimorphisms have been found in the hippocampus with males having larger cell bodies of pyramidal cells in the CA1 and CA3 regions than females (Isgor and Sengelaub, 1998; Romeo et al., 2004).

Gender differences in responses to stress have been proposed to be mediated by HPA and hypothalamus-pituitary-gonadal (HPG) axes (Fig. 1). In fact, men and women subjected to psychosocial stress, show differences in on HPA axis activation and pro-inflammatory cytokine production-induced by corticosteroids. These stress response differences have been attributed to changes in hormones levels induced by the menstrual cycle in women. In an early study, 18 women in the luteal phase of their menstrual cycle and 27 men were exposed to a psychosocial stress test (Trier social

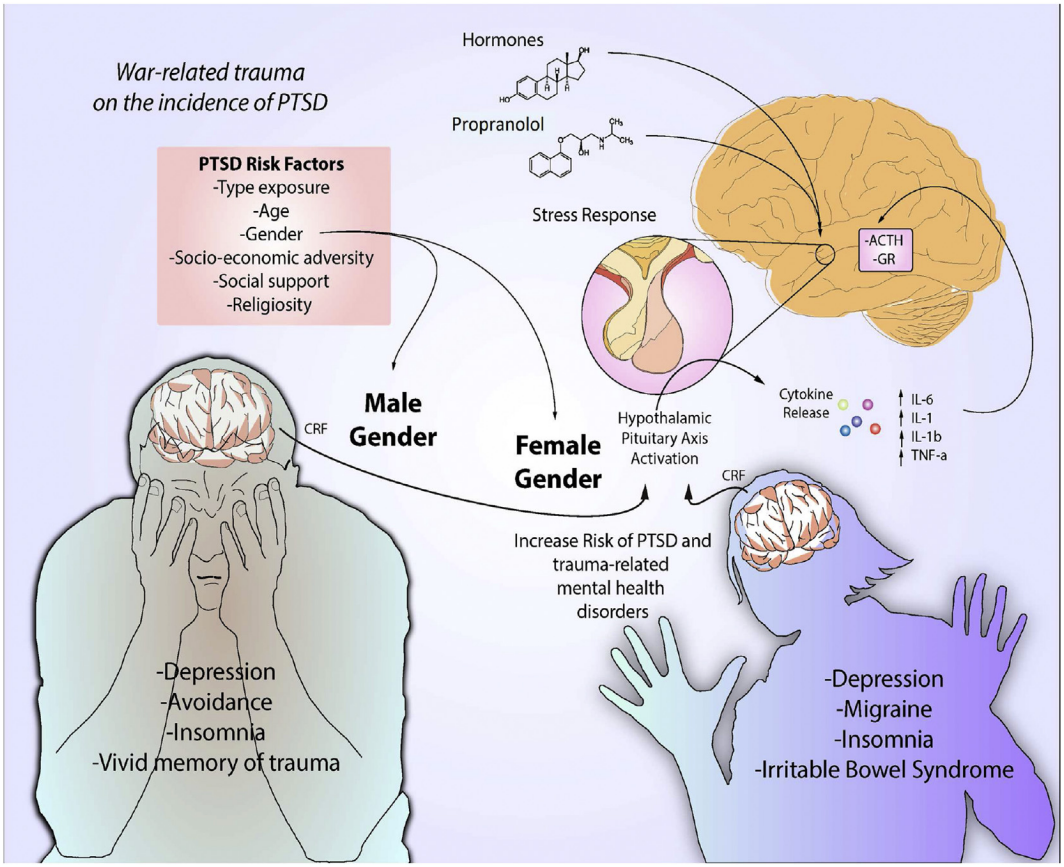

Fig. 1. Gender differences in the development of PTSD. Evidence suggests that during PTSD development, an increased release of corticotrophin releasing factor (CRF) induces an abnormal activation of the HPA axis and release of cytokines. In turn, cytokines such as IL-6, IL-1 and TNF-α can regulate stress responses by modulating glucocorticoid receptor (GR) and adrenocorticotropic hormone (ACTH) release. Evidence suggests that women have higher incidence of depression, migraine, insomnia and PTSD than men counterparts. This different susceptibility is attributed to hormonal and immune factors. Studies show that hormones such as estrogen, progesterone and dehydroepiandrosterone influence PTSD development by controlling the release of ACTH and activation of GR as well as the immune responses to psychological stress.

stress test) and salivary free cortisol levels were measured after exposure to stress (Rohleder et al., 2001). The stress paradigm induced significant increases in salivary free cortisol with no significant differences between men and women. In contrast, lipopolysaccharide (LPS)-stimulated cytokine production showed large gender-linked differences. They found that LPS-induced cytokine production decreased in response to stress in men but increased in women (Rohleder et al., 2001). The authors concluded that gender may exert differential effects on the immune system by modulating glucocorticoid -induced pro-inflammatory cytokine production (Rohleder et al., 2001).

1.1.2.3. Gender differences in the response to propranolol. Gender differences in the response to propranolol to prevent PTSD have been reported (Nugent et al., 2010). In a randomized double-blind 10-day trial, 29 injured patients (10–18 years of age) at risk for PTSD were started in propranolol or placebo treatments within 12 h post admission and continuously for 6 weeks. During this time PTSD symptoms and heart rate were assessed. Although intent-to-treat analyses showed no group differences, a significant interaction between gender and treatment was found (Nugent et al., 2010). Girls treated with propranolol reported more PTSD symptoms when compared to girls treated with placebo. On the contrary, boys receiving propranolol reported a no significant decrease in symptoms of PTSD when compared to boys treated with placebo (Nugent et al., 2010).

1.1.2.4. Effect of sex hormones on stress hormone responses

1.1.2.4.1. Preclinical studies. Preclinical data showed that females were more resilient to stress in helplessness behavior paradigms (Dalla et al.,). It has been speculated that higher basal corticosteroid levels found in female when compared to male rodents may explain these results (Iwasaki-Sekino et al., 2009; Viau et al., 2005).

The HPA axis and the hypothalamic-pituitary-gonadal (HPG) axis reciprocally influence each other. Gonadal steroids and their metabolites have been implicated in sex differences in fear and anxiety induced by stress. Sex hormones such as Testosterone, that is a product of the HPG, in addition to its sex hormone function has also modulatory effects over mood. Some studies have examined the effects of predator scent stress and gonadal hormones on associative trauma memory and anxiety behavior in male rats (Fenchel et al., 2015). The behavioral effects of immediate or delayed treatment with testosterone, testosterone receptor antagonist (flutamide) or vehicle on anxiety in male rats, were evaluated using the elevated plus-maze to measure anxiety, acoustic startle response to measure arousal levels and trauma-cue response. In addition they investigated changes in the expression of androgen receptor (AR) and estrogen receptor (ER) α, induced by predator stress. They found that rats exhibiting high levels of stress response presented a significant decrease in the expression of AR and ERα in the hippocampus. Immediate treatment with flutamide or delayed treatment with testosterone significantly decreased the stress response (Fenchel et al., 2015).

It is also known that estrogen has an effect on the release of stress hormones, prolonging ACTH secretion by impairing glucocorticoid receptor (GR)-mediated negative feedback (Redei et al., 1994; Young et al., 2001). Weiser and Handa reported that estradiol induced a disruption of GR-mediated negative feedback in the paraventricular nucleus of the hypothalamus (PVN) by acting on the estrogen receptor alpha (ERα) (Weiser and Handa, 2009). Although this effect may not be the only mechanism involved in gender differences in stress responses, as in another study it was found that ovariectomized female rats still showed high HPA axis response to stress, independent of circulating gonadal steroid levels. This evidence suggests the existence of other factors modulating gender-mediated differences in the HPA axis response to stress or an indirect effect during development. For example, early influences of sexual hormones on the central nervous system may also cause differential morphological (Patchev et al., 1995), physiological and behavioral responses (Mitev et al., 2003a, b). For example, neonatal estrogenization of female rats profoundly affects several regulatory substrates of the HPA axis, including the expression of corticotropin releasing hormone CRH, arginine-vasopressin (AVP) and GR in the brain, and their responsiveness to estrogen (Patchev et al., 1995). These changes can affect the responses to stress in adulthood or adolescence. Also in males, there is evidence reported that sexual hormones play a role in stress responses (Handa et al., 1994, 2013, 2011; Handa and Weiser, 2014). For example, it has been shown that in male hamsters, testosterone administration stimulated ACTH secretion and consequently, increased both adrenal steroid release and hepatic metabolism of cortisol (Handa and Weiser, 2014; Gaskin and Kitay, 1970, 1971).

Also stress regulate steroid hormones release. A study in the subject showed that foot shock or exposure to a novel open field increased plasma ACTH and corticosteriod, which was significantly greater in castrated than intact rats. Furthermore, castration enhanced and androgen treatment suppressed the stress-induced increase in ACTH and corticosterone levels via an androgen receptor-mediated mechanism (Handa et al., 1994). Furthermore, it was shown that the effect of castration can be inhibited by administration of the non-aromatizable androgen, dihydrotestosterone, thus implicating androgen receptors in this effect. In addition, following castration, due to removal of androgen-dependent repression, an increase in CRH in the PVN cells was observed (Bingaman et al., 1994). The authors suggested that testosterone may be inhibiting the HPA response.

Sex hormones also influence the expression and activity of the neuroplasticity factor the brain-derived neurotrophic factor (BDNF), which is fundamental to prevent the neurotoxic effect of chronic stress on synaptic function and memory (Carbone and Handa, 2013). Classical fear conditioning is a model of PTSD that permits to investigate the acquisition, storage and extinction of fear and the etiology and treatment of PTSD and other related anxiety disorders such as phobias. Also, sex hormones derivatives may have a modulatory effect over stress. For example, the metabolite of progesterone, allopregnanolone (ALLO), is a positive allosteric modulator of the GABAA receptor target of the benzodiazepines and like these drugs, ALLO has anxiolytic effects. ALLO levels fluctuates in females across the reproductive cycle. A study showed that the infusion of ALLO in the bed nucleus of the stria terminalis in male rats suppressed contextual fear response after fear conditioning, without affecting tone conditioned stimulus (CS). In addition, intra-BNST infusion of either finasteride (FIN), an inhibitor of ALLO synthesis, or 17-phenyl-(3α,5α)-androst-16-en-3-ol, an ALLO antagonist, in female rats enhanced contextual freezing. This evidence strongly suggests that sex differences in fear and anxiety may be the result of steroid modulation of brain regions involved in stress response. The authors also suggest that the susceptibility-resilience of women to develop PTSD after trauma may be linked to cyclic changes in neuroactive steroid activity within brain regions of the fear circuitry (Nagaya et al., 2015; Nagaya and Maren, 2015).

1.1.2.4.2. Clinical studies. Changes in hormonal levels have been identified as a factor mediating gender and age differences in resilience to stress (Shansky et al., 2009a; Kavushansky and Richter-Levin, 2006; Shansky, 2009, 2015; Shansky et al., 2009b, 2004, 2010; Shansky and Lipps, 2013; Shansky and Morrison, 2009; Shansky et al., 2006). In this respect, it has been found evidence that dehydroepiandrosterone sulphate (DHEA-S) levels were lower

in individuals with PTSD (Usta et al., 2016). Furthermore, in a clinical study with 397 traumatized patients showed that blood levels of DHEA-S after trauma, predicted the appearance of PTSD symptoms. PTSD symptoms appearance correlated with higher DHEA-S levels and a diminished cortisol/DHEA-S ratio at 6-week post-trauma (Mouthaan et al., 2014). Sexual hormones have a marked influence on brain function and behavior under physiological and pathological conditions. A decreased level of prolactin is associated to situations of stress induced by abnormal levels of dependency to authorities or individuals. In animal models has been found that prolactin response to stress was decreased in gonadectomized males compared with sham-operated and gonadectomized dihydrotestosterone (DHT)-treated rats (Bingaman et al., 1994).

In PTSD, highly vivid intrusive memories cause significant distress. New studies have identified several biological factors involved on the recollection and persistence of emotionally charged memories. Especially useful has been the investigation of biological and cognitive responses to negative and neutral images induced by stress. Using this paradigm, the potential contributions of sex hormones (estrogen and progesterone) and stress hormones (noradrenaline and cortisol) to the appearance and frequency of intrusive memories have been indexed. In one of these studies, half of participants of a total of 55 (29 women and 26 men) underwent a cold pressor stress (CPS) at the same time as viewing the images, while the control participants immersed their hands in warm water (Cheung et al., 2013). Participants viewed a series of negatively arousing and neutral images. Saliva samples were used to measure changes induced by stress in estrogen, progesterone, noradrenalin and cortisol levels. In addition, participants completed recall and intrusions thoughts two days later. Negative images resulted in greater recall and more intrusions than neutral images. In the cold water condition test, females recalled fewer neutral memories than males (Cheung et al., 2013). Higher estrogen levels were associated with a high number of intrusive recalls of negative images in women. While increase in cortisol was associated with diminished recall of negative memories in males (Cheung et al., 2013).

Recent findings suggest that the consolidation of emotional memories and number of spontaneous intrusive recollections (SIRs) of them are influenced by menstrual phases in females (Altemus et al., 1997; Ferree et al., 2011). In the mid-luteal phase, progesterone and cortisol levels are increased and this increase correlates with the enhancement of emotional memory. Women in the luteal phase of the menstrual cycle report more SIRs than men after exposure to emotional charged images (Felmingham et al., 2012a). In a different study, 30 women in the mid-luteal phase (high-progesterone group) and 26 women in non-luteal phases (low-progesterone group) were exposed to a series of neutral or negatively charged images followed immediately by either a cold pressor stress or control condition. After two days, participants were tested for image recollections and salivary cortisol levels. High progesterone level was associated with higher baseline and stress-evoked cortisol levels as well as enhanced recall of negative images. Also, a positive correlation was found between stress-induced increase in cortisol levels and the recall of threatening images. These authors suggested that progesterone enhanced cortisol responses to stress and memory recall for emotionally arousing stimuli (Felmingham et al., 2012a, 2012b).

Levels of circulating 17-β estradiol (E2) in women have been linked to deficits in fear extinction and extinction recall. A recent study investigated the effect of hormone status during stress exposure on fear acquisition and fear extinction (Antov and Stockhorst, 2014). In a 2-day experimental design, healthy participants, were subjected or not to psychosocial stress, and the interaction between natural E2 levels and fear conditioning, fear acquisition, immediate extinction (day 1), and 24 h-delayed extinction recall (day 2) were examined. The groups consisted in a) women in the early follicular phase (EF) of their menstrual cycle (low E2, low progesterone plasma levels), b) women in the mid-cycle phase (MC, high E2, low progesterone), and c) men. Conditioned responses were assessed measuring differential skin conductance responses. They data revealed an interaction between stress exposure and natural E2-status in women only. They found that MC-women, showed enhanced extinction recall on Day 2 (24 h after initial extinction training). In EF-women, the inverse was true. The authors concluded that extinction recall of conditioned fear acquired after stress depends on estrogen status in women. Therefore, they also suggest that exposure therapy, a type of psychotherapy based in fear extinction, in free-cycling female anxiety patients should take cycle status into account.

Altogether, the results from a variety of studies are consistent with the idea that gonadal steroid hormones may influence the release of stress hormones, neuronal function and fear memory mechanisms of storage, consolidation and recall.

1.1.2.5. Gender effects on the prevalence of combat PTSD. Differences by gender in the susceptibility to PTSD in subjects exposed to combat is still a controversial topic, with studies showing different outcomes. Most studies related to women and PTSD have been focused in the deleterious effects of military sexual trauma in the development of PTSD. In a study including more than 3000 women veterans, 23% reported a history of military sexual assault (MSA) and in this group the prevalence of depression was 3 times higher than in women without MSA (Goldzweig et al., 2006). It has been described that sexual stress is 4 times as important as war stress for the development of PTSD among female veterans (Fontana and Rosenheck, 1998; Fontana et al., 2000, 1997).

A number of previous studies have evaluated gender associated risk for developing PTSD, most of them found that women were more likely to develop PTSD and associated mental health conditions than men. (Goldzweig et al., 2006; Wolfe et al., 1999; Pierce, 1997; Engel et al., 1999; King et al., 1999).

At contrast, a recent longitudinal study, followed US military members for about 7 years from baseline during two follow-up periods between 2001 and 2008. Analyses were stratified by combat experience during follow-up. Outcome measures included a positive screen for PTSD and severity scores measured by the PTSD patient checklist-civilian version. From a total 4684 subjects matched by sex, 6.7% of women and 6.1% of men developed PTSD during follow-up. Results did not show significant differences for developing PTSD or for its severity among women and men. Furthermore, gender differences in hippocampal volume associated with PTSD or the risk to develop combat PTSD were not found (Jacobson et al., 2015). The authors concluded that women do not have a significantly different risk for developing combat PTSD than men (Jacobson et al., 2015). The different outcomes can be related to dissimilarities in psychological training, genetic backgrounds, homecoming environments and testing (self-report versus clinically administered tests) in the veterans populations studied.

On the other hand, the effect of deployment to a combat zone on plasma testosterone levels, and its relationship with the development of PTSD has been investigated (Reijnen et al., 2015; Karlović et al., 2012). One study found higher levels of testosterone in serum of patients with PTSD than controls (Karlović et al., 2012). In contrast, morning plasma testosterone levels were not found to differ in another study (Spivak et al., 2003). Recently, a longitudinal study investigated effect of deployment on testosterone in a total of 918 male subjects (Reijnen et al., 2015). Participants were evaluated for testosterone levels prior to deployment to a combat zone in Afghanistan and after 1 and 6- month post-deployment. Also, the

association with PTSD symptoms reported at 1 and 2-year post-deployment was determined. The data showed higher levels of plasma testosterone after deployment than under basal pre-deployment conditions. Moreover, pre-deployment low testosterone levels predicted the development of PTSD symptoms at 1 and 2-year post-deployment. This evidence suggests that testosterone may represent a biological vulnerability factor for the development of PTSD. No correlation between cortisol levels and changes in testosterone were found (Reijnen et al., 2015).

1.1.3. Role of innate immune system in PTSD

1.1.3.1. Preclinical studies. Profound physiological and neurochemical changes underlie the clinical symptoms of PTSD. Despite the role of the central immune system on PTSD has not been clearly elucidated, early evidence showed a relationship between this disorder and the alteration of the immune system. In this regard, several preclinical studies have shown that high levels of stress induce an exacerbation in the expression of pro-inflammatory markers such as cytokine interleukin (IL)-1β in both plasma and brain. IL-1 plays various roles in inflammatory responses (Fantuzzi, 2001) and cerebral IL-1 is important mediating several basal brain such as sleep, social behavior and body temperature (Opp et al., 1988). The cerebral expression of the pro-hormone IL-1, IL-1-converting enzyme, the endogenous IL-1 receptor antagonist, and its receptors have been well documented (Cunningham and De Souza, 1993; Cunningham et al., 1991, 1992). IL-1 has been easily detected in CNS after conditions leading to neuroinflammation (Minami et al., 1991; Mogi et al., 1996, 1994). However, detection of IL-1 in brain under non-pathological conditions has proven to give variable results. Under pathological conditions cerebral infusion of IL-1 produces fever (Ansel et al., 1987), hyperalgesia (Opree and Kress, 2000), slow-wave sleep (Chang and Opp, 2000), reduced food and water intake (Opara et al., 1995; Plata-Salaman and Ffrench-Mullen, 1992), reduced social interaction (Barnum et al., 2008) and induced alterations in peripheral immune parameters (Sundar et al., 1990; Brown et al., 1992). Thus, IL-1 may play a role in mediating the neurochemical and behavioral consequences of stressors.

Few studies have investigated the effect of psychological stress on cerebral and plasma IL-1 content. However, it has been reported preclinical evidence that immobilization stress increased both IL-1 mRNA expression and activity in animals (Shintani et al., 1995a). Furthermore, it has been observed that intracerebroventricular infusion of an antagonist of the IL-1 receptor prevented stress-enhanced fear learning (SEFL) (Jones et al., 2015). Likewise, the infusion of an antagonist of the IL-1 receptor blocked both the activation of the HPA axis and the increase of monoamine neurotransmitters levels in the hypothalamus induced by immobilization stress in rodents (Shintani et al., 1995a). Similarly, using another experimental paradigm of stress named stress-enhanced fear learning (SEFL) in rats, it was found that severe stress induced an increase in IL-1β expression within the hippocampal formation, but not in the amygdala or the perirhinal cortex (Shintani et al., 1995b).

In addition to IL-1β, it has been found that the expression of various pro-inflammatory markers is increase by psychological and oxidative stress, both of which negatively impact brain function and behavior. For example, in one study, rats were subjected a single prolonged stress (SPS) and chronically treated with the NADPH oxidase (NOX2) inhibitor apocynin either early or in a delayed manner. The different groups were tested for the expression of TNFα, IL-1β, IL-6, IL-10, malondialdehyde, superoxide dismutase, NOX2, 4-hydroxynonenal and parvalbumina (PV) in the hippocampus (Liu et al., 2015). The results showed that stressed rats presented enhanced fear conditioning, and higher anxiety levels than control non-stressed rats. These behavioral changes were accompanied by augmented expression of 4-hydroxynonenal, IL-6, malondialdehyde, NOX2, and decreased PV expression. Inhibition of NOX2 with apocynin reversed all these abnormalities after SPS (Liu et al., 2015). The authors concluded that oxidative stress and neuroinflammation induced by NOX2 inhibition resulted in PV interneuron loss in the hippocampus which in turn triggered the PTSD symptoms (Liu et al., 2015).

1.1.3.2. Clinical studies. Confirming a relationship between psychiatric disorders and neuroinflammation, current epidemiological evidence shows a correlation between having an altered immune function within the central nervous system and suffering of mental health conditions such as bipolar disorder, depression and PTSD. The alteration involves changes in neurotransmission and glucocorticoid functions as well as an enhanced expression of cytokines such as IL-1β, IL-6 and the tumor necrosis factor-alpha (TNFα) (Jones and Thomsen, 2013). This increase in stress and immune factors parallels with memory deficits, altered social behavior and fear responses (Jones and Thomsen, 2013).

Another insightful study assessed changes in DNA methylation in the promoter of several immune system-related genes from blood collected pre- and post-deployments from U.S. soldiers (n = 75) with and without PTSD (n = 75) via pyrosequencing (Rusiecki et al., 2013). The promoter regions analyzed included the insulin-like growth factor 2 (IGF2), IL8, IL16, and IL18. They found significant differences in the change of methylation pre-to post-deployment between cases and controls. In the deployed personnel, those who did not develop PTSD had lower methylation levels of H19 and IL18 after deployment, whereas those who did develop PTSD had higher levels of IL18. The differences in IL18 methylation seems to be dependent on genetic factors as the effect was more pronounced among older and Caucasian male soldiers and those with shorter deployments (6–12 months) than soldier with other genetic backgrounds.

A more recent and extensive prospective longitudinal genome association study (GWAS), investigated genetic linkage associated with PTSD development in combat-exposed U.S. Marines and Sailors scheduled for deployment to Iraq and/or Afghanistan (N = 3494). This study was designed to identify risk and resiliency factors for combat-induced stress symptoms. Participants were assessed using the Clinician-Administered PTSD Scale (CAPS) and diagnosed using the DSM-IV diagnostic criterion. From the 3494 trauma-exposed males, 940 were diagnosed with PTSD. The GWAS meta-analysis identified several genes that positively correlated with PTSD development including the phosphoribosyl transferase domain containing 1 gene (PRTFDC1) as a main genome-wide PTSD locus and other genes related to immune response such as JAK 1 and FASLG (Nievergelt et al., 2015). Previous genetic (Guffanti et al., 2013) and gene expression analysis (Glatt et al., 2013), also indicated a relationship between changes in those genes and susceptibility to PTSD.

Additional studies, have revealed an association between high incidence of infections, autoimmune diseases and pathological changes of immune cells with the diagnosis of PTSD. A study published by Yehuda and colleagues studied gene expression from whole blood in 35 subjects exposed to the World Trade Center attack (Yehuda et al., 2009). The subjects with PTSD (n = 15) presented significant differences from the controls in several genes linked to immune function and HPA activity (Binder et al., 2008).

More recently, PTSD-associated differences in a subset of immune cells in blood have been reported. In one of these studies, they investigated the frequency of subtypes of NK cells in plasma obtained from combat-exposed male war veterans, with PTSD (n = 67) or without PTSD controls (n = 72). The results showed that compared to controls subjects, veterans with PTSD had a

significantly higher frequency of an atypical population of $CD56^{-}CD16^{+}$ NK cells and lower frequency of the functional CD56brightCD16-NK cells. Furthermore, patients with PTSD presented lower levels of cortisol and higher levels of catecholamine in plasma than controls.

On the other hand, it is known that IL-6 secretion is suppressed by glucocorticoids and stimulated by catecholamines, thus as expected, IL-6 concentrations in CSF have been found higher in patients with PTSD than in control subjects (Baker et al., 2001). Paradoxically, changes in IL-6 levels were not found in the plasma from PTSD patients. The authors proposed that high levels of IL-6 in CSF may reflect neurodegeneration or compensatory neuroprotection (Baker et al., 2001).

The activation of the immune system is also considered a potential factor inducing stress sensitization. It is known that soldiers exposed to combat stress, may experience an increase in PTSD symptoms when exposed to stressful life events (SLE), a form of stress sensitization. Some evidence suggests that immune activation, expressed as enhanced cytokines leukocytes upon stimulation, may underlie stress sensitization (Smid et al., 2015). For example, recently an investigation of SLE and PTSD symptoms up to two-year post-deployment and the production of mitogen-induced cytokine by leukocytes at one and 12 months in soldiers returned from Afghanistan ($N = 693$) was carried out. The results revealed significant interaction effects among combat stress exposure, cytokine production, and post-deployment SLE on PTSD symptoms over the first 2- year post-deployment. In subjects exposed to high combat stress, both high mitogen-stimulated T-cell cytokine production and innate cytokine production correlated with higher PTSD symptoms in response to post-deployment SLE but no in soldiers exposed to low combat stress or low cytokine production.

High stimulated T-cell and innate cytokine production may contribute to stress sensitization in recently deployed, high combat stress exposed soldiers. These findings suggest that detecting and eventually normalizing immune activation may potentially complement future strategies to prevent progression of PTSD symptoms following return from deployment.

1.1.4. Role of sex hormones with the immune system in psychological stress and PTSD

1.1.4.1. Preclinical studies. As previously discussed, abundant evidence suggests that stress contributes to the dysregulation of the inflammatory response and severity of inflammatory diseases. However, the contribution of sex and gonadal hormones on these changes have been not extensively investigated in animal models of PTSD. Nonetheless, some preclinical studies have explored the effect of sex on the changes in immune response induced by stress. One of these studies investigated the effect of sound stress on the function of polymorphonuclear neutrophil-immune cells at preclinical level by examining the effect of stress on the production of reactive oxygen species (ROS) and phagocytosis by rat neutrophils. Peripheral blood neutrophils were collected from female and male rats exposed to intermittent sound stress (over 4 days). Stress suppressed ROS production in males (but not females) and neutrophil phagocytosis in both sexes. All these effect were suppressed in adrenal medullectomizated rats. To investigate the role of sex hormones in these differential response, rats were gonadectomized prepubertally and exposed to stress as adults. In gonadectomized males, stress produced an even larger decrease in ROS production, but had no effect on the stress-induced inhibition of phagocytosis. Gonadectomy prevented the stress-induced inhibition of neutrophil phagocytosis in females. These data indicate that the adrenal medulla, perhaps via release of epinephrine, suppresses neutrophil ROS production in males and phagocytosis in males and females.

1.1.4.2. Clinical studies. As discussed before, PTSD patients exhibit dysfunction of the innate immune inflammatory and neuroendocrine systems. It is increasingly clear that changes in both systems are interrelated and simultaneously contributing to the etiology of PTSD (Pace et al., 2012). To understand this relationship a pilot clinical study investigated the activity of the pro-inflammatory transcription factor Nuclear factor-κB (NF-κB), which control cytokines expression, in blood mononuclear cells obtained from women ($n = 12$, 19–48 years of age) with child abuse-related PTSD and 24 age-matched non-PTSD controls. Glucocorticoid sensitivity of monocytes was assessed determining the concentration of dexamethasone required to suppress lipopolysaccharide-induced tumor necrosis factor-alpha production by 50% (DEXIC50). Women with PTSD displayed higher NF-κB pathway activity compared to controls and the level of NF-κB activity positively correlated with PTSD severity. NF-κB pathway activity was also associated with higher DEX IC50 (i.e. decreased sensitivity of monocytes to glucocorticoids) across all participants (Nishi et al., 2015).

Responses to hormones and changes in the immune system also seems to differ according to gender in PTSD patients. One good example is the response to oxytocin investigated as a therapeutic tool for prevention of PTSD. An exploratory study aimed to clarify the relationships between the oxytocin level and physical and psychosocial factors by gender in accident survivors. Investigated, blood samples of 235 and 155 survivors at baseline, and at one month after the accident, respectively. Spearman's correlation and univariate and multivariate regression analyses were used to examine the relationships between the serum oxytocin levels and PTSD symptoms by gender. They found that in men, the oxytocin levels were negatively associated with C-reactive protein at baseline and did not predict any psychological variables at the 1-month follow-up. In contrast, in women, the oxytocin levels were positively correlated with cooperativeness at baseline and predicted seeking social support, positive reappraisal, accepting responsibility and problem solving at the follow-up.

The oxytocin levels were not associated with PTSD, depression, and anxiety symptoms. This study suggests that the effect of oxytocin in posttraumatic coping and inflammation differs by gender in accident survivors. Gender differences might be a key consideration when developing interventions using oxytocin.

The analysis of mononuclear blood cells using new genetic techniques is considered a powerful tool to assess changes in inflammatory markers associated to PTSD. One of this genetic studies used gene microarrays on $CD14^{+}$ monocytes to investigate inflammatory activity and to identify new signaling pathways dysregulated in PTSD in both female and male subjects (Neylan et al., 2011). Participants included, 49 men (24 with PTSD and 25 age-matched trauma-exposed PTSD controls) and 18 women (10 PTSD and 8 age-matched controls). The data show a general down-regulation in the expression of monocyte genes in this group however, the data did not support the occurrence of chronic inflammation in male PTSD subjects. In contracts, preliminary data from the female PTSD group showed a gender-specific increase in the activity of immune factors. The authors suggested that differential patterns of monocyte gene expression in PTSD, suggest a gender dimorphism in biologic pathways activated in PTSD.

A genome-wide association study (GWAS) from few years ago, also linked genomic changes associated with gender-linked factors influencing heritable susceptibility to develop PTSD (Guffanti et al., 2013). They studied two different cohorts with different genetic background the first included primarily African American women and a second cohort that mainly comprised European American women. They genotyped 413 women from the first group - 94 PTSD cases and 319 controls exposed to traumatic events for >700,000

markers and tested 578 PTSD cases and 1963 controls from the second cohort. A network-based analysis integrating data from GWAS-derived independent regions of association and the Reactome database of functional interactions. The network-based analysis indicated that the more relevant results were enriched for factors linked to immune function and telomere maintenance as well as changes in specific microRNA named lincRNA.

2. Conclusions

Altogether current evidence supports an important role of inflammation and sex hormones on the behavioral and neurofunctional aspects of PTSD. Gender differences in the prevalence of stress disorders may be explained by a differential modulation by sex hormones of the immune system and brain responses to stress in male and female subjects. However, in the complexity of PTSD, age and other environmental and cultural factors may greatly influence the effect of stress hormones on the development of PTSD. Further studies, correlating sex hormones, gender and environmental influences on the development of PTSD are required. A better understanding of the role of these factors may lead us to more effective preventative or healing therapies.

Conflict of interest

Drs. Valentina Echeverria, George Barreto, Marco Ávila-Rodriguez and Cristhian Mendoza have no actual or potential conflict of interests concerning the topic in the present review.

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Artículo N°2

Intranasal cotinine improves memory, and reduces depressive-like behavior, and GFAP + cells loss induced by restraint stress in mice.

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Intranasal cotinine improves memory, and reduces depressive-like behavior, and GFAP + cells loss induced by restraint stress in mice

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ABSTRACT

Posttraumatic stress disorder (PTSD), chronic psychological stress, and major depressive disorder have been found to be associated with a significant decrease in glial fibrillary acidic protein (GFAP) immunoreactivity in the hippocampus of rodents. Cotinine is an alkaloid that prevents memory impairment, depressive-like behavior and synaptic loss when co-administered during restraint stress, a model of PTSD and stress-induced depression, in mice. Here, we investigated the effects of post-treatment with intranasal cotinine on depressive- and anxiety-like behaviors, visual recognition memory as well as the number and morphology of GFAP + immunoreactive cells, in the hippocampus and frontal cortex of mice subjected to prolonged restraint stress. The results revealed that in addition to the mood and cognitive impairments, restraint stress induced a significant decrease in the number and arborization of GFAP + cells in the brain of mice. Intranasal cotinine prevented these stress-derived symptoms and the morphological abnormalities GFAP + cells in both of these brain regions which are critical to resilience to stress. The significance of these findings for the therapy of PTSD and depression is discussed.

1. Introduction

Although the effects of acute stress are usually brief and can be overcome quickly, exposure to chronic or extreme forms of unescapable stress can lead to neurochemical, morphological and functional changes in the brain that have been associated with posttraumatic stress disorder (PTSD) (North et al., 2016) and major depressive disorder (MDD) (Nestler et al., 2002). These stress-induced psychiatric diseases are characterized by symptoms such as intrusive distressing thoughts, anhedonia, irritability (McHugh et al., 2012), feelings of guilt, sleep disorders (Williams et al., 2015), cognitive impairment (Jak et al., 2016), anxiety (Wilder Schaaf et al., 2013) and sometimes treatment-resistant depression (Stander et al., 2014). Furthermore, these conditions have been associated with functional and structural changes in several regions of the brain including the amygdala (Laugharne et al., 2016), entorhinal cortex, prefrontal cortex and hippocampus (Meng et al., 2016; Sheynin and Liberzon, 2016; Yoon et al., 2017; Zhu et al., 2016). At cellular level, PTSD and depression are also associated with a decrease of glial fibrillary acidic protein (GFAP) immunoreactive cells ($GFAP^+$) in the brain (Saur et al., 2016; Nestler et al., 2002; Fuller et al., 2010). GFAP is a family of proteins that includes eight isoforms expressed by different subpopulations of astrocytes as well as immature brain cells. These isoforms include $GFAP^{+1}$, GFAP delta and GFAP kappa. GFAP delta appears to be linked with neural stem cells (NSCs) and may be involved in migration.

Furthermore, the expression of GFAP has been reported to decrease in response to microgravity (Day et al., 1998). Another predominantly-astroglial enzyme, glutamine synthase, has been reduced in the frontal cortex following intraventricular injection of aluminum (Guo-Ross et al., 1999), which paralleled alterations in GFAP expression. These results

Abbreviations: ANOVA, Analysis of variance; BDNF, Brain derived neurotrophic factor; CS, Conditioned Stimulus; EPM, Elevated plus maze; GDNF, glial derived neurotrophic factor; GABA, Gamma-Amino Butyric acid; ΓΣΚ3β, Glycogen synthase kinase 3 beta; HPA, Hypothalamus-pituitary-adrenal; MD, Major depression; min, Minutes; NOR, Novel object recognition; OF, Open field; NGF, neurotrophic growth factor; nAChR, nicotinic acetylcholine receptor; PBS, Phosphate buffered saline; PFC, Prefrontal cortex; PT, Porsolt's test; PTSD, Post-traumatic stress disorder; RS, Restraint stress; RT, Room temperature; *sec*, Seconds; 5-HT, Serotonin; TBS, Tris-buffered saline; TBST, TBS with 0.1% Tween 20; SSRIs, Selective serotonin reuptake inhibitors.

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suggest an impairment of astrocytic responsivity in frontal cortex following toxic insults. Stress-induced depression is associated with a reduction of neurogenesis, neuroinflammation and a decrease of astrocytes in the brain (Sanacora and Banasr, 2013), and reduced GFAP expression has been found associated with schizophrenia, bipolar disorder and depression (Cobb et al., 2016; Webster et al., 2005). In addition, dysfunction of GFAP expression has been reported in encephalopathy (Kretzschmar et al., 1985). Astrocytes have been implicated in brain and neuronal functions supporting learning and memory and emotional responses (Dienel, 2017; Gibbs et al., 2006; Lee et al., 2014). Astrocytes have been identified as playing a significant role in maintaining brain homeostasis and supporting neuronal function (Weinstein et al., 1991). Coherent with these roles, it has been found a dysfunction of astroglia in the hippocampus of subjects with depression (Cobb et al., 2016) and other psychiatric and neurological conditions (Saur et al., 2016).

Because astrocytes support neuronal function and neurogenesis, it is thought that a decrease in astrocytic function is a critical factor underlying maladaptive responses in individuals with posttraumatic stress disorder (PTSD). Interestingly, treatment with antidepressants such as fluoxetine improved mood and induced a restoration of astroglia, suggesting that a restoration of astrocytes function is associated with changes in behavior.

An excellent model to investigate PTSD-induced, treatment-resistant depression (TRD) is the prolonged restraint stress paradigm (Perrine et al., 2016). Using this model, it has been shown that chronic stress induces cognitive deficits and depressive-like behavior, and morphological changes such as a decrease in the length and number of dendrites and synapses in neurons of the CA3 region of the hippocampus (Watanabe et al., 1992). Indeed, it has been shown that a two-hour immobilization stress combined with forced swim stress induced a decrease in the number of astrocytes in the hippocampus of rats (Imbe et al., 2012). Chronic restraint stress (6 h/day for 3 weeks), but not short-term restraint stress (6 h/day for 3 days), caused mechanical hypersensitivity and aggressive behavior. The chronic restraint stress induced a significant decrease of GFAP protein levels in the ventrolateral periaqueductal gray (Imbe et al., 2012). This decrease in astrocyte immunoreactivity (IR) was accompanied by a parallel decrease in glutamate transporter EAAT2 expression. The EAAT2 protein levels in the 3 week-stress group was significantly lower (−20%) than in the control group. In contrast, there was no significant differences in the GFAP and EAAT2 protein levels between the three-day stress groups control in the periaqueductal gray matter (Imbe et al., 2012). It is thought that a deficit in glutamate transport after chronic stress may trigger neuronal dysfunction due to excitotoxicity in the brain.

Cotinine, a positive modulator of the α7 nicotinic acetylcholine receptors (nAChRs) (Echeverria et al., 2016b), has shown to decrease anxiety and to improve the extinction of fear in rodents subjected to fear conditioning a model of PTSD (de Aguiar et al., 2013; Zeitlin et al., 2012). Furthermore, continue treatment with oral cotinine prevented working memory loss and depressive-like behavior, as well as increased synaptic density in the hippocampus and frontal cortex of mice subjected to chronic immobilization stress (Grizzell et al., 2014a, 2014b). Cotinine has a long plasma half-life (19 to 24 h) and shows minor side effects in humans (Grizzell and Echeverria, 2015). Cotinine also has anti-inflammatory properties, overriding the production of cytokines that are under transcriptional control of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) system (transforming neurotrophic factor alpha (TNF-α), Interleukin (IL)-1β, IL-6, IL-12, IL-23) (Rehani et al., 2008).

The targets of cotinine are the most abundant nicotinic receptors in the brain, the low affinity (α7) and high affinity (α4β2) nAChRs (Colquhoun and Patrick, 1997). The activation of α7 receptors at the synapse triggers an increase in permeability to Na^+ and Ca^{2+} ions (Pichon et al., 2004). These currents depolarize the cell and activate different neuronal signaling cascades (Exley and Cragg, 2008). Furthermore, Na^+ and Ca^{2+} currents depolarize the pre-synaptic membrane, inducing the release of neurotransmitters such as dopamine, serotonin, glutamate, and gamma-amino butyric acid (GABA) (d'Incamps and Ascher, 2014; Exley and Cragg, 2008). In this way, the stimulation of the α7 and α4β2 receptors in the prefrontal cortex can promote the activation of glutamatergic neurons that normally inhibit the activity of the amygdala after chronic stress (Bencherif et al., 2014; Broide and Leslie, 1999). Thus, by acting on these receptors, cotinine may alleviate TRD in patients with PTSD.

Intranasal (IN) administration of CNS drug is acquiring increasing attention because of therapeutic advantages in reducing time of action of drugs, decreasing systemic effects and preventing hepatic first-pass elimination (Hanson and Frey, 2007, 2008). In this study, in the search of new delivery methods for cotinine, we tested the behavioral effects of IN cotinine on depressive-like behavior, anxiety and working visual recognition memory when administered after prolonged restraint stress. In addition, to understand the mechanism of action of IN cotinine, we also examined its effect on the levels of $GFAP^+$ cells in the hippocampus and frontal cortex of mice subjected or not to restraint stress. The significance of these results for the treatment of PTSD and TRD is discussed.

2. Materials and methods

2.1. Animals

Mice were obtained from the animal facilities of the University of Chile, and maintained with free access to commercial food and water, in a controlled environment with an average temperature of 22 °C under a 12 h/12 h dark/light schedule. C57BL/6 male mice weighing between 20 and 30 grams (g) and aged about 2–3 months were used. Mice were acclimatized to the housing facility for a week before experiments. Test and animal care were performed according to protocols approved for the Universidad San Sebastian ethical committee and performed in compliance with the Guide for the care and use of Laboratory Animals adopted by the National Institute of Health (USA). Mice were weighed twice a week during the performance of the experiments and until euthanasia.

2.2. Drugs and reagents

Cotinine ((5S)-1-methyl-5-(3-pyridyl)-pyrrolidin-2-one) and other miscellaneous reagents were obtained from Sigma-Aldrich (Saint Louis, MO) unless stated otherwise.

2.3. Experimental groups and drug treatments

Mice between 2 and 3 months of age after one week of acclimatization were randomly divided into two groups. Stressed mice were subjected to restraint stress. Control (non-restrained) mice were allowed to move freely during this period. After the stress exposure period, mice were divided into three experimental groups: 1) Non-restrained mice treated with vehicle (PBS, pH 7.4) serving as unstressed controls ($n = 8$); 2) Restrained mice (RS) treated with vehicle ($n = 8$); 3), RS mice treated with 24 μl of a cotinine solution (10 mg/ml in PBS, pH 7.4) via intranasal route of administration ($n = 6$). Treatments were administered daily until euthanasia. After two weeks of treatments mice were behaviorally tested and euthanized (Fig. 1).

2.4. Awake intranasal cotinine delivery

Intranasal delivery was performed as previously described (Hanson and Frey, 2007). Mice at 2–3 months of age were hand-restrained, positioned in a supine position, and administered two 12 μl drops of cotinine solution (10 mg/ml in PBS), or PBS alone, into both nares. Mice were given an extra 12 μl treatment drop if the mouse expelled out the

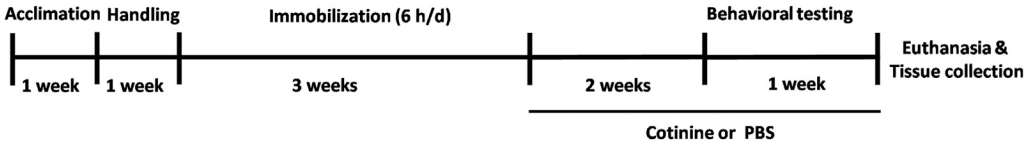

Fig. 1. Diagram representing the experimental design. Male mice ($n = 6$–8/condition) were housed and habituated to their cages before restraint stress or resting conditions were applied. After this period mice were treated, behaviorally tested and euthanized. IHC analysis was then performed in selected brain regions of the mice.

solution. Mice were kept in the supine posture for 5 seconds (*sec*) after delivery to facilitate the delivery. The administration was performed daily until euthanasia. Mice were subjected to behavioral testing about 2 h after their morning dose. Mice were euthanized using cervical dislocation by a well-trained investigator.

2.5. Restraint stress

The stress paradigm was performed as previously described (Grizzell et al., 2014a). Briefly, mice were immobilized inside transparent 50 ml conic transparent tubes. Tubes permitted only subtle movements of the mice and contained holes in both ends to allow normal animal breathing. Mice were immobilized for 6 h/day for 21 days at <300 lx.

2.6. Behavioral analysis

Mice were tested for locomotor activity and working memory using the open field (OF) and Novel object recognition (NOR) tests, respectively. Depressive-like behavior, and anxiety were tested in the forced swim (depressive-like behavior) and the elevated plus maze (EPM) (anxiety) tests, respectively. Animal behavior was recorded and analyzed using the Any - maze® software (Stoelting CO, USA).

2.6.1. Open field test (OF)

OF was conducted to monitor locomotor activity as described (Zeitlin et al., 2012). Mice were individually placed in an uncovered square arena (40 cm × 40 cm × 35 cm), allowed to freely explore for 30 min (min) while monitored with a video tracking software (ANY-Maze, Stoelting Co, Illinois, USA) under moderate lighting. Several parameters including total distance travelled, speed, and time spent in the center and peripheral zones (20 cm × 20 cm) were measured to assess locomotor activity.

2.6.2. Forced swim test

The forced swim test (FST) is a reliable and extensively used test to measure the effect of antidepressants (Naitoh et al., 1992). We have previously shown that this test is reliable to test stress-induced depression after restraint stress as follow. Mice were placed in a transparent cylinder filled with water at 25 °C for 5 min and behavior was recorded. After a brief period of strong activity, rodents adopt a characteristic immobile posture. Immobility is defined as the time the mouse was engaged in only the minimal movements required for breathing and to keep the head above the water.

2.6.3. Novel object recognition (NOR, visual recognition memory test)

The NOR test permits investigators to determine short- and long-term recognition memory, as well as motivation for novelty (Antunes and Biala, 2012; Grayson et al., 2015; Yang et al., 2015). Cognitive enhancement in these tests has been reported using α7nAChRs agonists and 5-HT antagonists (Antunes and Biala, 2012).

The NOR test starts with a habituation step that consists in putting each mouse to freely explore an open and empty testing arena (40 cm × 40 cm × 35 cm) for 10 min. On the next day, each mouse was placed in the same arena but containing two identical objects located equidistant to each other (familiarization phase) and led to freely explore the objects for 5 min. Then, mice were put back to their cages and permitted to rest for 30 min. After this time, each mouse was placed back in the arena containing one of the old objects that were present during the familiarity phase, and a new object. The time exploring the objects was recorded during 5 min in both steps. Exploratory behavior was normalized for animal activity by calculating the exploration index (EI) that corresponds to the time spent by the mouse exploring the new object/total time spent with both objects) × 100%. The software Any-Maze (Stoelting Co.) coupled to a recording camera and computer systems was used for behavioral recording and documenting.

2.7. Morphological analyses of GFAP immunoreactive cells in the hippocampus of mice

2.7.1. Brain tissue preparation

For all protein analyses, mice were euthanized and brains removed. Each brain was divided into two hemispheres. The left hemisphere of brains was dissected out to collect the regions of interest and quickly frozen for later analyses. For the immunohistochemical (IHC) analysis the right hemisphere of each mouse brain was placed in 10% formalin in PBS pH 7.4 for 48 h and then embedded in paraffin. Each region of interest was located using Paxinos Atlas as a reference (Franklin and Paxinos, 2001), and serial cortices of 4 μm ($n \geq 5$/mouse) were collected using the Microtome Leica RM 2125RT and mounted on silanized glass slides.

2.7.2. GFAP$^+$ cells immunohistochemical analysis

The analysis of GFAP$^+$ cells was performed using tissue slices containing the ventral hippocampus (Approx. Bregma −4.08 mm, interaural 4.92 mm) and frontal cortex (Approx. Bregma 3.2 mm, interaural 1.54 mm). Sagital sections of brains were collected in PBS and processed for GFAP immunoreactivity (IR). Brain slices were immersed in xylene and a decreasing graduation of ethanol baths for hydration. Then, slides were subjected to a standard process of antigenic recovery in buffer citrate pH = 6 in a pressurized saucepan (Biocare Medical, Walnut Creek, CA) for 30 min. Next, slides were incubated with a solution of 3% hydrogen peroxide to block endogenous peroxidase for 5 min, washed with PBS, and blocked with a horse serum solution (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, USA) for 10 min at room temperature (RT). Sections were washed in PBS and incubated for 1 h (Franklin and Paxinos, 2001) at RT with an antibody against GFAP 1:100 (Sigma). After washing with PBS, sections were incubated with a biotinylated secondary antibody for 10 min. Then, sections were washed with PBS and incubated with the amplifier solution from the Vectastain Elite kit for 10 min at RT. The reaction was visualized using ImmunoDetector DAB (SB Bio Inc., Santa Barbara, CA, USA). For counterstaining, sections were stained with hematoxylin for 30 *sec*, dehydrated in baths of ascending percentages of alcohol solutions and xylene, and mounted with synthetic resin.

For the IR analysis, for each mouse, three digital images were randomly selected at 40× magnifications in the areas of interest (hippocampus and frontal cortex) ($n = 5$–6/condition). The images were taken using a digital camera attached to a light microscope (Micrometrics, MilesCo Scientific, Princeton, MN, USA) connected to a camera operated by a commercial software (Micrometrics SE Premium). The determination of the area of the immunolabeling was calculated delimiting the IR areas using the ImageJ software (National Institute of Health, Bethesda, MA, USA). For all analyses, GFAP$^+$ astrocytes were

selected randomly from the frontal cortex and the CA1, CA3 and dentate gyrus regions of the hippocampus and quantified. Using a digital camera on an inverted microscope, black and white images of GFAP$^+$ astrocytes were obtained and processed with Image J software. Using a 20× objective, cells were chosen randomly in the same area selected for immunostaining, and the binary overlay of a cell was created by thresholding. For all images, a threshold value was established at the level at which the binary overlay entirely enclosed the cell body and projections. All pixels above the threshold value were considered as belonging to the cell images. Finally, the binary silhouette of the whole cell was reduced to its one-pixel outline for estimation of the fractal dimensions with the FracLac 2.5 ImageJ plug-in (Karperien et al., 2013; Karperien and Jelinek, 2015).

2.7.3. Quantitative fractal analysis

Fractal analysis was done on binary images by means of the dilation method (Schaffner and Ghesquiere, 2001). The slope of the regression line (S) is related to the fractal dimension (D) by $D = 1 - S$. Each pixel in the cell outline was replaced with a disk of a diameter fluctuating from 3 to 61 pixels and the area of the widened outline divided by the diameter of structuring element was plotted against this diameter on a log-log scale.

Parameters calculated included:

Cellular area: The area of the cell body that is calculated as the two-dimensional cross-sectional area contained within the boundary of the cell body.

Arbor area: The area of the convex polygon formed by connecting the tips of the longest astrocytic processes (convex hull area). Convex hull values indicate the size of the branching field of the astrocyte. The amount of physical space is defined in terms of convex-hull volume, surface area, area, and or perimeter.

Lacunarity: Measures heterogeneity and complements fractal dimension analysis in describing structural complexity (Karperien et al., 2013; Karperien and Jelinek, 2015; Schaffner and Ghesquiere, 2001).

3. Statistical analysis

To analyze differences between-groups means in the behavioral and immunohistochemical studies, the following were used. Student's *t*-tests or Kruskal-Wallis were used when comparing two conditions and when comparing three or more levels of a factor, one-way followed by Tukey's or Tukey-Kramer post hoc tests (where applicable) or a repeated measure, 3 × 3 factorial ANOVA (treatment condition × brain region) followed by Fisher's LSD post-hoc tests were used where appropriate. For the IHC analyses of hippocampal subregions, each mouse brain contained several GFAP+ cells which were then averaged across subject by region and included in the analyses as mouse being the foci of analyses. When individual cells were used as the unit of focus in the analyses, the results were similar. All statistical analyses were performed with the software GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 24 (IBM, Armonk, NY, USA). Differences were considered statistically significant for p-value < 0.05.

4. Results

4.1. Effect of posttreatment with intranasal cotinine on stress-induced changes in locomotor activity

To assess changes in locomotor activity related to restraint stress and cotinine treatment, we first tested each mouse in the open field, a task that permits investigators to assess changes in locomotor activity and anxiety behavior. One-way ANOVA analysis revealed significant differences between treatment groups in locomotor activity, expressed as distance travelled in the OF test ($F(2,17) = 5.144$, $p = 0.018$). A Tukey Post-hoc analysis indicated a significant increase ($p < 0.05$) in locomotor activity in the PBS-treated restrained mice when compared to PBS-treated control mice (Ctrl + PBS: 32 ± 18 meters (m) vs RS + PBS: 61 ± 19 m, $p < 0.05$) (Fig. 2A). Restrained mice treated with IN cotinine showed lower values of distance travelled than vehicle-treated restrained mice (RS + PBS: 61 ± 19 m vs RS + Cot: 49 ± 13 m) and that were no significantly different from the nonstressed control group ($p < 0.05$) (Fig. 2A).

4.2. Effect of intranasal cotinine on stress-induced changes in depressive-like behavior

It has been previously shown that chronic immobilization stress is associated with depressive-like behavior in rodents (Ferraz et al., 2011). The time mice spent immobile in the forced swim test is a measure of depressive-like behavior in rodents (Karl et al., 2003; Naitoh et al., 1992). We have shown that oral cotinine administered before and continuously to restrained C57BL/6 mice, substantively decreased depressive-like behavior induced by stress (Grizzell et al., 2014a). Similarly, in this study we found significant differences in depressive-like behavior between treatment groups ($F(2, 13) = 8.848$, $p = 0.004$). A post hoc Tukey test revealed that following post-treatment with IN cotinine, the restrained mice showed a significant decrease in immobility in the forced swim test ($p < 0.05$). However, cotinine-treated restrained mice showed immobility values not significantly different from controls, but significantly lower than PBS-treated restrained mice ($p < 0.01$) (Fig. 2B).

4.3. Effect of intranasal cotinine on stress-induced cognitive impairment

To analyze whether intranasal cotinine can revert the stress-induced deterioration in cognitive abilities, we tested the effect of post-treatment with intranasal cotinine on short-term recognition memory in mice. In the familiarization phase, one-way ANOVA analysis revealed no significant changes between groups in the time expend exploring the equal objects ($F(2,12) = 0.3422$, $p > 0.05$) or entries to the area of each object ($F(2,12) = 0.738$, $p > 0.05$). However, restraint stress and cotinine induced significant changes in cognitive abilities in this task in the time spent with the new object ($F(2,15) = 7.755$, $p < 0.01$), as well as the number of entries in the second object area ($F(2,14) = 3.756$, $p < 0.05$). A Tukey post hoc analysis showed that stressed mice showed a reduction in their cognitive abilities expressed as a decrease in the EI for the new object when compared to control mice ($p < 0.01$). Cotinine-treated stressed mice showed better discrimination for the new object showing a significantly higher number of entries to the novel object area when compared to the vehicle-treated stressed mice ($p < 0.05$). Also, cotinine-treated stressed mice showed an increase in the exploration index when compared to vehicle-treated stressed mice but the difference did not reach statistical significance (Fig. 3).

4.4. Analysis of GFAP immunoreactivity and GFAP$^+$ cells morphology

GFAP+ cells in both hippocampus and frontal cortex possessed a distinct morphology in between groups (Fig. 4). The immunohistochemistry analysis of GFAP+ IR cells showed significant differences in GFAP IR between treatment groups in the hippocampus ($F(2, 15 = 49.08$, $p < 0.001$) (Fig. 4A and B) and frontal cortex (One way ANOVA, $p < 0.001$) (Fig. 4A and C). GFAP IR was found dramatically reduced in both the hippocampus (−55%, $p < 0001$) (Fig. 4A) and frontal cortex (−87%, $p < 0.0001$) of the vehicle-treated restrained mice, when compared to the vehicle-treated nonstressed control mice (Fig. 4B). However, cotinine administered after the RS almost completely restored GFAP IR in the hippocampus (84% of control value, Fig. 4B) and frontal cortex (90% of control values, Fig. 4C).

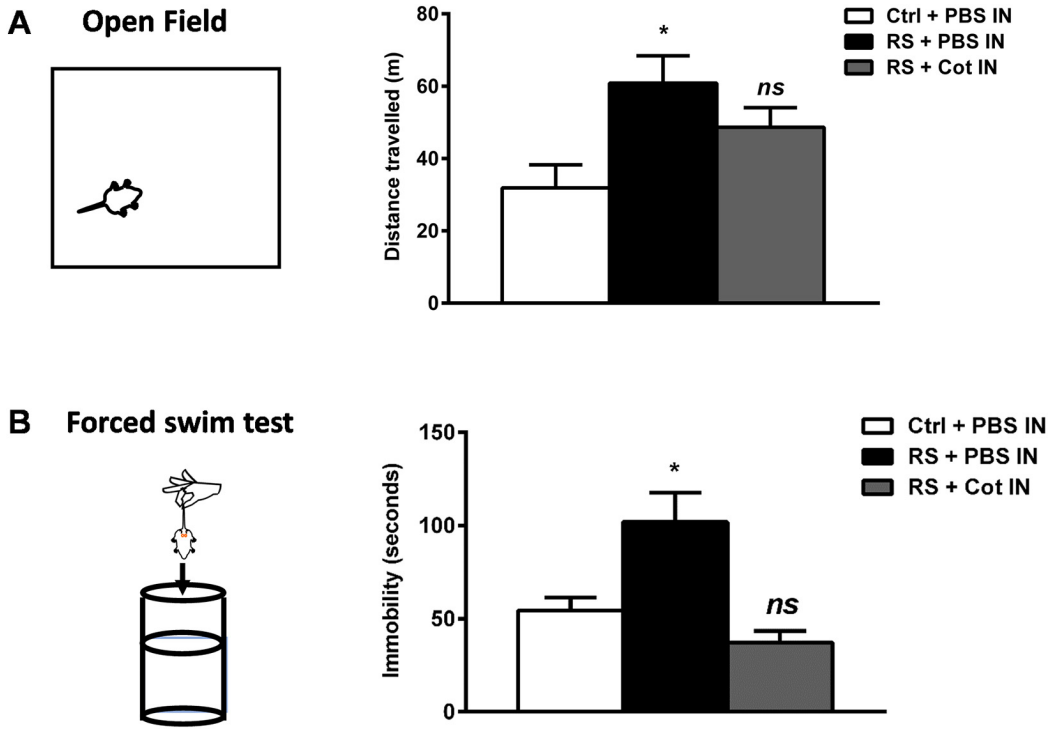

Fig. 2. Effect of intranasal cotinine on locomotor activity and reducing depressive like behavior after chronic restraint stress. Figures to the left represent the behavioral tests used. The graphs depict the effect of restraint stress (RS) and intranasal cotinine (Cot) on locomotor activity in the open field (A), and depressive-like behavior in the forced swim test (B). ns, non-significant change; *, $p < 0.05$.

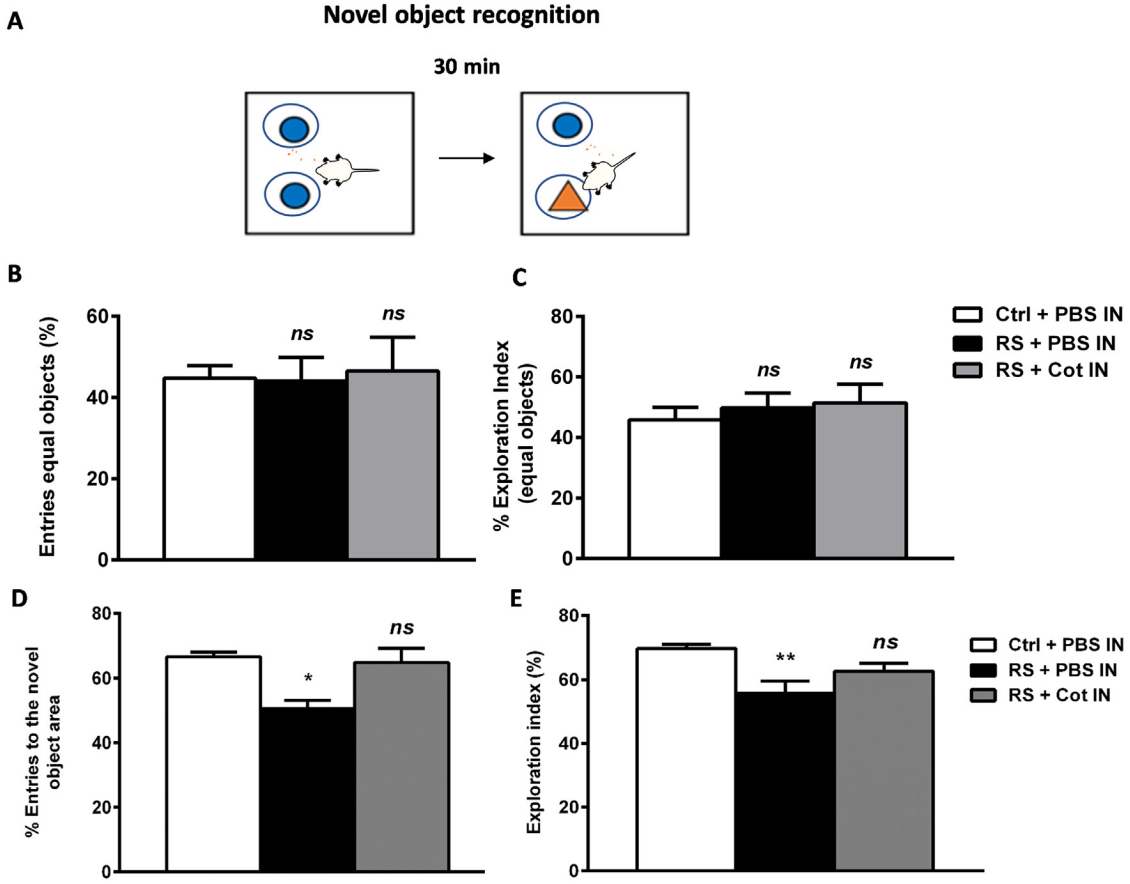

Fig. 3. Effect of intranasal cotinine on visual recognition memory after restraint stress. Control (Ctrl) and restrained (RS) mice were treated with intranasal (IN) cotinine 10 mg/ml in PBS (Cot) or vehicle (PBS) for and visual recognition memory were tested in the NOR test. Data is expressed as the percentage of control values and represents the mean ± SEM ($n = 4$–5 mice). **, $p < 0.01$. ***, $p < 0.001$.

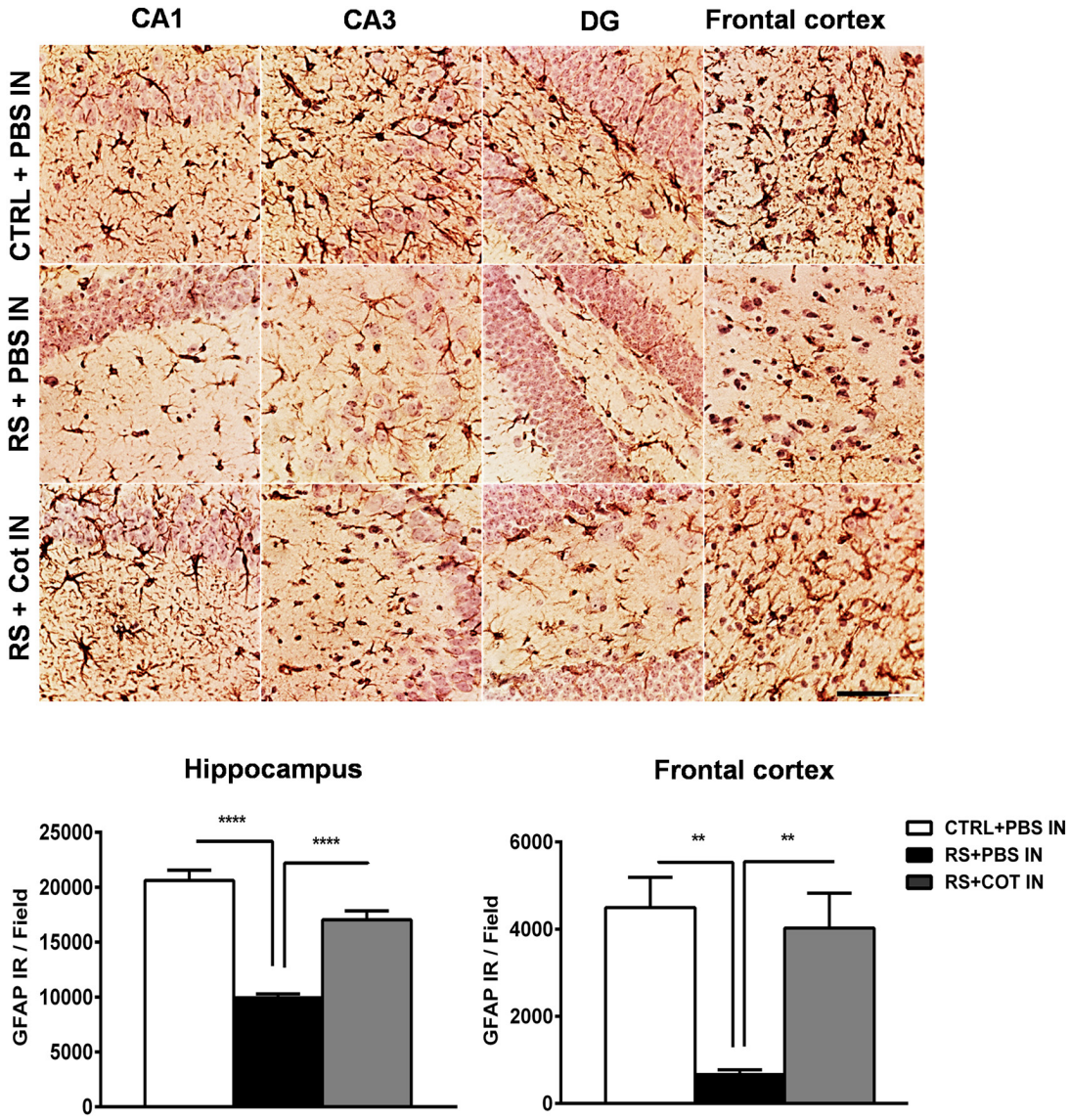

Fig. 4. Effect of cotinine on GFAP expression after chronic stress Glial fibrillar acidic protein (GFAP) IR in the frontal cortex (FC) and hippocampus of mice. The images to the right depict the negative control of immunostaining (Ctrl (−)). GFAP IR in control mice treated with intranasal (IN) PBS (CTRL + PBS); mice subjected to restraint stress (RS) and treated with PBS IN and intranasal cotinine (10 mg/ml) (RS + Cot). Each bar represents the average of the percentage of immunostaining for each group field. From left to right the bars represent the mean ± standard deviation. Data was analyzed using One-way ANOVA. **, $p < 0.01$; ***, $p < 0.001$.

4.4.1. GFAP$^+$ cells density

One-way ANOVA analyses of GFAP$^+$ cells were performed in randomly selected quadrants of three sections per mouse. The number of hippocampal GFAP+ cells counted varied according to mice treatments (Ctrl,144; RS, 97; RS + Cot, 140). The analyses showed that mice subjected to RS showed a significant decrease in the number of astrocytes in the hippocampal regions analyzed when compared to nonstressed control mice (Fig. 5). Similar results were obtained when the frontal cortex of mice was analyzed. However, these abnormalities were corrected by intranasal cotinine treatment. One-way analyzes of cell counting of sections immunoassayed for GFAP IR revealed a significant effect of treatments on the number of GFAP$^+$ cells in the CA1 ($F(2, 7) = 43.20$, $p < 0.001$), CA3 ($F(2, 6) = 13.86$, $p < 0.001$) and DG regions ($F(2, 6) = 12.92$, $p < 0.001$). A multiple comparison test revealed a significant reduction in cell density in the CA1, CA3 and DG regions of restrained mice when compared to control mice (CA1, $p < 0{,}001$; CA3, $p < 0.01$; DG, $p < 0{,}05$), respectively. Furthermore, GFAP$^+$ cell density was significantly higher in the cotinine-treated restrained mice relative to vehicle-treated restrained mice (CA1, $p < 0{,}01$; CA2, $p < 0{,}05$; DG, $p <$

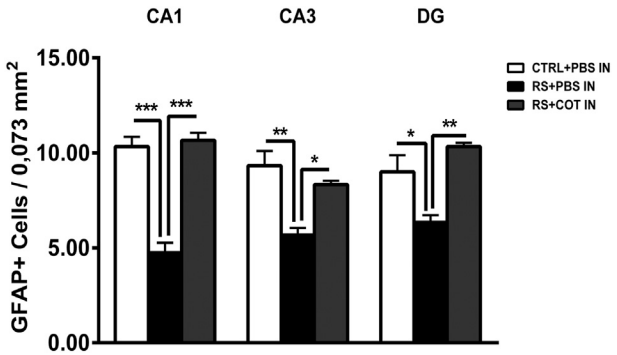

Fig. 5. Changes in GAFP$^+$ cells in the hippocampal formation. Plots represent the number of GFAP+ cells in the different brain regions in Controls non-stressed (CTRL + PBS IN) mice and a reduced staining intensity in the stressed mice (RS) treated with IN PBS compared to control (CTRL+ PBS IN) and restrained mice treated with IN Cotinine (24 μl, 10 mg/ml)(RS + Cot IN).

0,01) (Fig. 5). No significant differences in GFAP$^+$ cell density was observed between vehicle-treated and cotinine-treated control mice.

4.4.2. Changes in the morphology of GFAP$^+$ cells induced by restraint stress and cotinine

In the stressed mice, GFAP$^+$ astrocytes in the hippocampi and frontal cortices showed different appearances depending on levels of stress and treatments. Small cells mostly with short, tiny and poorly ramified processes were observed in the vehicle-treated restrained mice. At the contrary, large GFAP$^+$ cells with longer and more complex arborization were observed in cotinine-treated restrained mice and nonstressed control mice (Fig. 6A). To evaluate these changes, randomly selected individual astrocytes from the brain areas of interest were analyzed for cell area, arbor area, fractal dimension, and lacunarity.

4.4.3. Effect of cotinine on cell area

A 3 × 3 repeated measures ANOVA (treatment condition × brain region wherein brain region is the within-subject factor) revealed a significant main effect of treatment condition in cell area across regions ($F_{(2,8)} = 19.755$, $p < 0.001$). Post-hoc analyses revealed that across the CA1, CA3 and DG hippocampal regions, the astrocytes of vehicle-treated restrained mice had significantly less cell area than both non-stressed controls as well as cotinine-treated restrained mice ($p < 0.05$). Furthermore, the hippocampal cell areas of cotinine-treated restrained mice were statistically indistinguishable from nonstressed controls (Fig. 6B).

4.4.4. Effect of IN cotinine arbor area

A 3 × 3 repeated measures ANOVA (treatment condition × brain region) of the arbor area (Hull) revealed significant main effects of both treatment condition ($F(2,8) = 18.166$, $p < 0.001$) and brain region ($F(2,7) = 4.777$, $p < 0.05$). Post-hoc analyses reveal that in astrocytes of the CA1, cotinine-treated mice had significantly more arbor area than the non-stressed controls ($p < 0.05$) and marginally more than their vehicle-treated, stressed counterparts ($p = 0.075$). In the CA3, cotinine-treated, restrained mice had significantly more arbor area of astrocytes than non-stressed controls ($p < 0.05$) and vehicle-treated, restrained mice ($p < 0.01$) (Fig. 6C). However, in the DG, both cotinine-treated and non-stressed controls had greater astrocytic arborization than vehicle-treated, restrained mice ($p < 0.001$) with no differences between cotinine-treated, stressed mice and unstressed controls ($p = 0.533$). Although there was a significant main effect of the within-group factor which suggested that levels of arborization differed between brain regions, no post-hoc tests were conducted as we felt this was not pertinent to our investigation.

4.4.5. Effect of IN cotinine lacunarity

A 3 × 3 repeated measures ANOVA (treatment condition × brain region) revealed a significant main effect of treatment condition in lacunarity ($F(2,8) = 5.067$, $p < 0.05$). Post-hoc analyses detected significant differences in the DG only wherein vehicle-treated, restrained mice had reduced lacunarity of astrocytes relative to both their cotinine-treated, stressed and vehicle-treated, non-stressed counterparts ($p < 0.05$; Fig. 6D).

4.4.6. Effect of IN cotinine fractal dimension

A 3 × 3 repeated measures ANOVA (treatment condition × brain region) of changes in fractal dimension (FD) revealed a significant main effect of treatment condition ($F(2,8) = 5.888$, $p < 0.05$). Post-hoc tests revealed that vehicle-treated, restrained mice had a significant reduction in FD of astrocytes in the DG when compared to cotinine-treated,

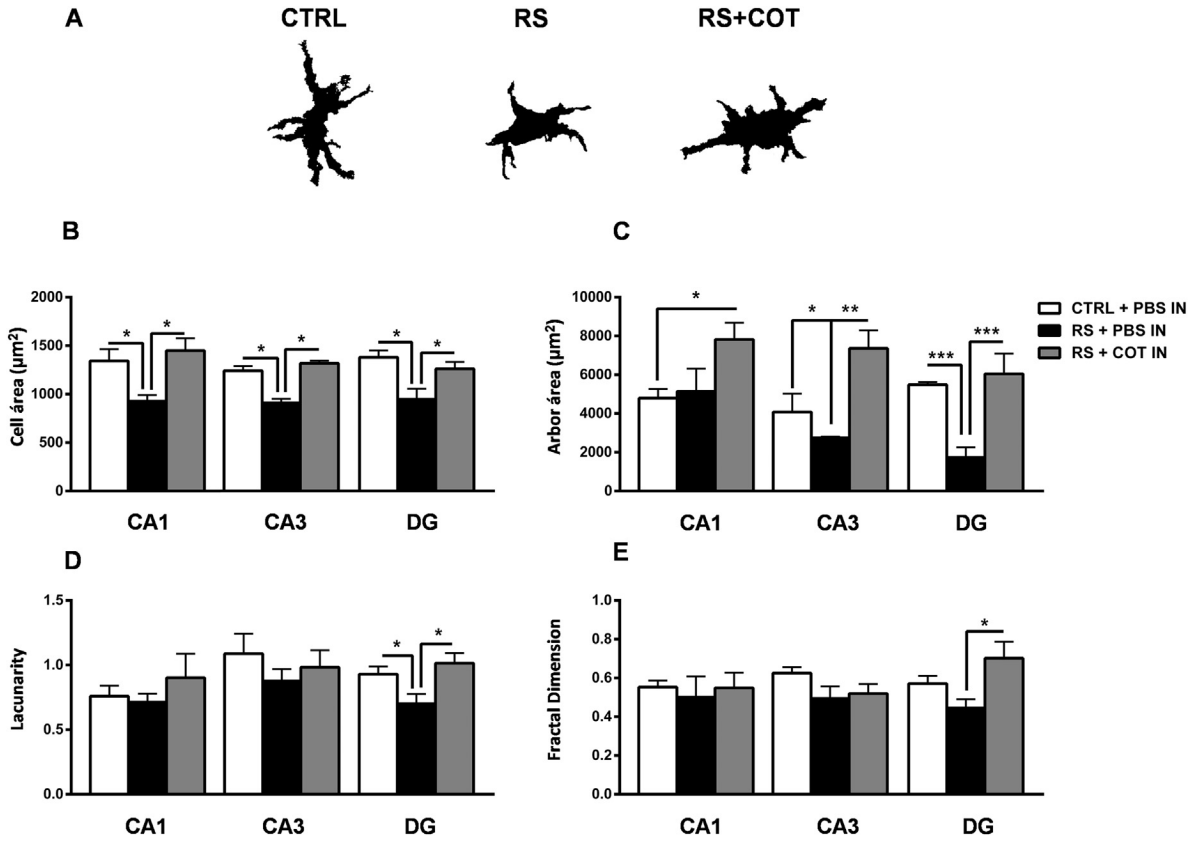

Fig. 6. Analysis of the effect of intranasal cotinine on cerebral neuronal cytoarchitecture in the hippocampus. Figure representing the changes in morphology of GFAP+ cells in the CA1 region of the Hippocampus of mice. Diagrams represent the GFAP+ cells area silhouettes; Graph depicting the changes in cell (B); Arbor area (C); Lacunarity (D), and Fractal dimension (E) in the hippocampus of Control (CTRL) or restrained (RS) mice treated with intranasal (IN) PBS (PBS), or IN cotinine (10 mg/ml)(Cot).

restrained mice ($p < 0.05$) as well as a marginal reduction in FD in the CA3 when compared to vehicle-treated nonstressed mice (Fig. 6E).

5. Discussion

Chronic stress in rodents is considered a good animal model to investigate antidepressants for treatment-resistant depression (TRD) in PTSD. In this work, the effects of post-treatment with IN cotinine on behavior and GFAP + cells in the hippocampus and frontal cortex of adult male mice subjected to stress were investigated. The results show that IN cotinine normalized the otherwise abnormal behavior in the chronically stressed mice. In addition, we found a clear effect of intranasal cotinine on normalizing the morphology and number of $GFAP^+$ cells in the hippocampus of restrained mice.

Therapeutic approaches for TRD in PTSD patients include treatment with combinations of anxiolytic, antidepressants, sedatives, antipsychotics drugs, and antiepileptic drugs as well as cognitive behavioral therapy (Heinrichs et al., 2013). These treatments, although can temporally reduce anxiety and depression, only a small percentage of patients shows remission and >75% maintains the diagnosis of PTSD and or depression at the end of treatments (Javidi and Yadollahie, 2012). Although, some progress has been made in defining biomarkers to predict the potential response to current treatments (Colvonen et al., 2017), new drugs or therapeutic strategies are required. Few new drug candidates (Lee et al., 2017) and other treatments such as transcranial magnetic brain stimulation and hypnotherapy (Rotaru and Rusu, 2016) are currently been tested (Trevizol et al., 2016).

We have shown that co-treatment with orally administered cotinine prevented depressive-like behavior in C57BL/6 mice subjected to immobilization stress (Grizzell et al., 2014a) and female rats subjected to chemotherapy treatments (Iarkov et al., 2016). However, the effect of oral or IN cotinine administered after chronic stress exposure has not been explored before. In the forced swimming test, cotinine almost completely normalized depressive-like behavior and restrained mice not treated with cotinine had immobility values significantly higher than mice post-treated with cotinine.

To define new treatments, it is important to target brain alterations associated with the pathological changes in brain functions. When GFAP + IR was assessed, it was found that RS caused a 55% and 87% decrease in $GFAP^+$ astrocyte IR density in the frontal cortex and the hippocampus, respectively. Cotinine restored $GFAP^+$ IR in both brain regions to control mice values. In addition, IN cotinine normalized the number and morphology of GFAP + cells, increasing the cell area and structural complexity and length of astrocytes projections in both brain regions studied. These findings agree with previous studies in rodent models of chronic stress showing a decrease in $GFAP^+$ cells in the hippocampus (Orlovsky et al., 2014; Santha et al., 2015). One of these studies showed that stress significantly reduced both the number and body cell volume of astrocytes (both approximately 25%), and that these phenomena correlated with a decrease in the volume of the hippocampal formation and prefrontal cortex. These changes were counteracted by treatment with the antidepressant fluoxetine (Czeh et al., 2007; Czeh et al., 2006; Fuchs et al., 2006; Lucassen et al., 2006). Based on this evidence, further studies have investigated the effect of therapeutic compounds over behavior and astrocyte function (Feng et al., 2015; Xia et al., 2013). Morphological changes of astrocytes may have a serious impact on both neuronal function and viability as astrocytes control the levels of extracellular glutamate, preventing excitotoxicity in the brain. Moreover, a prominent decrease in astroglia has been found in the brain of patients that suffered from major depression disorder (MDD). However, the type of astrocyte pathology in MDD is distinctive from the observed in other neurological and neurodegenerative disorders such as epilepsy (Babb et al., 1996; Webster et al., 2017), traumatic brain injury (Kabadi et al., 2014; Villapol et al., 2014), stroke (Hennessy et al., 2015), amyotrophic lateral sclerosis (Nagai et al., 2007; Radford et al., 2015; Yamanaka et al., 2008), Huntington's disease (Crotti and Glass, 2015; Kim et al., 2015), Parkinson's disease (Liu et al., 2015; Niranjan, 2014) or Alzheimer's disease (Fuller et al., 2010; Li et al., 2011; Ugbode et al., 2017; Winkler et al., 2015). In these disorders, glial scar formation occurs in parallel to astrogliosis, although a protective role of astrocytes has been also suggested in these conditions (Benarroch, 2005; Forster and Reiser, 2016; Otani et al., 2006; Spence et al., 2011; Stobart and Anderson, 2013; Verkhratsky et al., 2013). In MDD there is no astrogliosis, as the expression of GFAP and other markers of astrocytes is decreased, revealing a different pathological mechanism.

Drugs that affect the cholinergic system may be future options for PTSD and depression. Currently several other cholinergic drugs have been tested for treatment resistant depression. Scopolamine, a muscarinic antagonist, has been tested in placebo-controlled studies with positive result (Szczepanik et al., 2016). On the other hand, the nAChRs antagonist mecamylamine has been tested as an augmentation for antidepressants without positive results (Moller et al., 2015).

Numerous studies have shown that cotinine, a modulator of the nAChRs, has beneficial effects on depressive behavior and synaptic plasticity in neurodegenerative and psychiatric conditions (de Aguiar et al., 2013; Echeverria et al., 2016a; Echeverria et al., 2016b; Gao et al., 2014; Grizzell and Echeverria, 2014; Grizzell et al., 2014a; Grizzell et al., 2014b; Grizzell et al., 2017; Patel et al., 2014; Terry et al., 2015; Wang et al., 2015; Wildeboer-Andrud et al., 2014; Zeitlin et al., 2012). The studies have tested the effect of oral doses of cotinine in animal models of pathology and behavior. However, as a potential clinical application, we explored intranasal delivery of cotinine thinking in its use as a fast delivery post-trauma therapy with reduced systemic side effects and costs. The results obtained in the present study show that cotinine helps to improve cognitive abilities, and decreased dramatically depressive-like behavior and anxiety after a week of intranasal administration of the drug dissolved in a saline solution.

Positive allosteric modulators of the nAChRs have been proposed as a drug with a novel approach with therapeutic possibilities for cognition, neurodegeneration and psychiatric conditions including PTSD. Unlike traditional nAChRs agonists, the MAP would enhance cholinergic function, but maintaining the natural temporal pattern of receptor stimulation, by endogenous agonists (Please see Fig. 7).

In addition, α7nAChRs are expressed in microglia and peripheral macrophages where their activation has anti-inflammatory effects. Thus, positive modulators of these receptors such as cotinine both in microglia and neurons can reduce neuroinflammation and promote neuronal survival and synaptic plasticity, respectively.

Cotinine increased the expression of GFAP in the hippocampus and frontal cortex of mice subjected to immobilization stress, suggesting that cotinine not only can prevent the pathological cellular changes induced by stress, but it can also help to the recovery of the brain, restoring brain functions and the expression of $GFAP^+$ cells in brain regions involved in memory formation and emotional and fear responses. A previous study showed that young mice with more complex astrocyte structures perform better in the object recognition test (Diniz et al., 2016). Recently, Lee et al. investigated whether the blockade of astrocytic vesicular release induced behavioral abnormalities. They found a significant impairment in recognition memory when tested in the NOR, and the authors proposed that astrocytes are necessary for novel object recognition behavior and to maintain functional gamma oscillations both in vitro and in awake-behaving animals (Lee et al., 2014). These results are coherent with our results showing that the amelioration of astrocytes function was associated with the improvement in recognition memory in the restrained mice.

A recent report showed a marked decrease in the soma area and length of astrocytes projections and reduced arborization induced by stress using fear conditioning with electric shock (Saur et al., 2016). The authors showed in a rat model of PTSD, that in the hippocampus, stress decreased the density of $GFAP^+$ astrocytes and negatively changed its morphology, diminishing the total number of primary processes, and their arborization complexity. Stress also altered the polarity

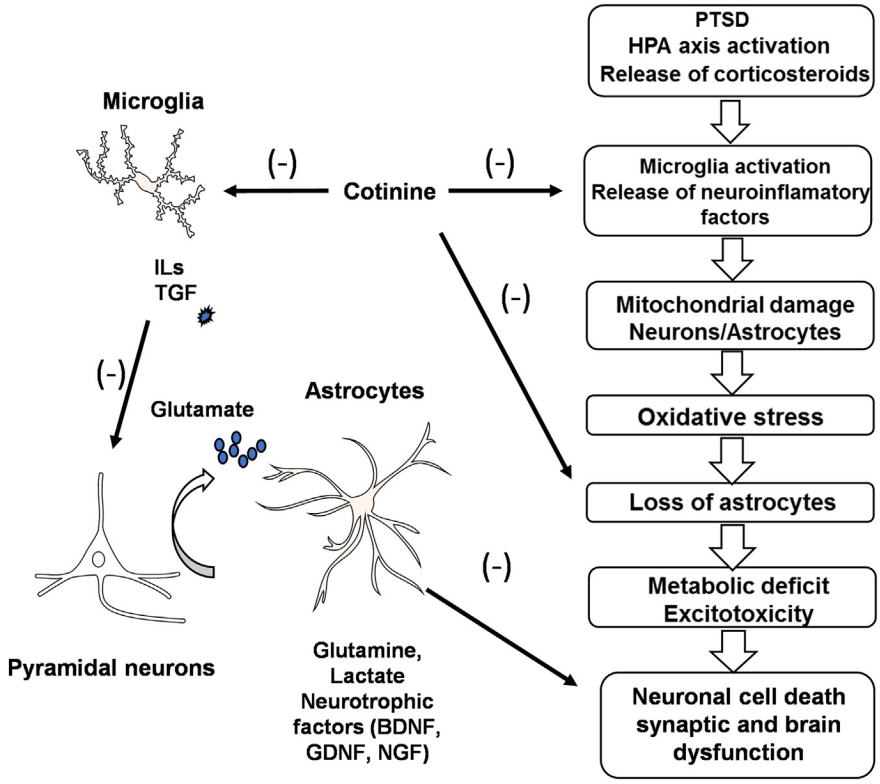

Fig. 7. Potential mechanisms of action of cotinine in reducing depressive-like behavior Microglia activation by stress is counteracted by cotinine, thus protecting GFAP+ cells from oxidative stress and apoptosis. This effect will recover the ability of astrocytes in supporting neuroplasticity by providing nutrients, energy molecules, neurotrophic factors and preventing excitotoxicity by up taking glutamate an excitatory neurotransmitter. BDNF, brain-derived neurotrophic factor; GDNF, glial derived neurotrophic factor; GLT1, glutamate transporter; HPA, hypothalamus-pituitary adrenal gland; IL, interleukins; NGF, nerve growth factor; TGF, Transforming growth factor.

of hippocampal astrocytes. No such changes were observed in astrocytes from the amygdala. Indeed, the fact that cotinine IN is also effective in diminishing the effects of stress suggests that the effects of oral cotinine is due to its direct effect in the brain, and not the effect of one of its metabolites or derivatives.

Numerous studies show the neuroprotective effect of the positive modulators of the α7 nAChRs (Balsera et al., 2014; Barreto et al., 2017; Barreto et al., 2015; Echeverria et al., 2016b). In our view, these results represent new mechanism of action of cotinine under chronic psychological stress and support the view that a positive modulation of the neuronal nicotinic receptors has restorative effects on the brain of subject suffering from PTSD. The results of this study help clarify the potential beneficial effects of cotinine in brain repair. We believe that these results are critical to better understanding of the clinical and therapeutic effects of cotinine on people suffering from neurodegenerative diseases and PTSD-associated conditions.

6. Conclusions

The evidence obtained in this study permits to conclude that post-treatment with IN cotinine is effective in restoring mood equilibrium and cognitive abilities as well as astrocytes function after chronic restraint stress in mice. The preceding constitutes the first evidence about the action of cotinine on $GFAP^+$ cells. This finding represents a new mechanism of action of cotinine to restore neuronal survival and plasticity after stress. The IN delivery of cotinine proved to be effective as a method of treatment with cotinine for PTSD or restraint stress-associated disorders. It is necessary to supplement the results presented in this work with further clinical research, enabling to establish whether the observed beneficial effects of cotinine in rodents are equally effective in humans.

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Artículo N°3

Intranasal Cotinine Plus Krill Oil Facilitates Fear Extinction, Decreases Depressive-Like Behavior, and Increases Hippocampal Calcineurin A Levels in Mice.

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Intranasal Cotinine Plus Krill Oil Facilitates Fear Extinction, Decreases Depressive-Like Behavior, and Increases Hippocampal Calcineurin A Levels in Mice

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Abstract
Failure in fear extinction is one of the more troublesome characteristics of posttraumatic stress disorder (PTSD). Cotinine facilitates fear memory extinction and reduces depressive-like behavior when administered 24 h after fear conditioning in mice. In this study, it was investigated the behavioral and molecular effects of cotinine, and other antidepressant preparations infused intranasally. Intranasal (IN) cotinine, IN krill oil, IN cotinine plus krill oil, and oral sertraline were evaluated on depressive-like behavior and fear retention and extinction after fear conditioning in C57BL/6 mice. Since calcineurin A has been involved in facilitating fear extinction in rodents, we also investigated changes of calcineurin in the hippocampus, a region key on contextual fear extinction. Short-term treatment with cotinine formulations was superior to krill oil and oral sertraline in reducing depressive-like behavior and fear consolidation and enhancing contextual fear memory extinction in mice. IN krill oil slowed the extinction of fear. IN cotinine preparations increased the levels of calcineurin A in the hippocampus of conditioned mice. In the light of the results, the future investigation of the use of IN cotinine preparations for the extinction of contextual fear memory and treatment of treatment-resistant depression (TRD) in PTSD is discussed.

Keywords Depression · Posttraumatic stress disorder · Fear extinction · Krill oil · Cotinine

Introduction

The failure to extinguish fear memories is one of the more pervasive symptoms of posttraumatic stress disorder (PTSD). It has been proposed that extinction recall deficits can be due to inability to process contextual information and to use it to modulate fear [1]. Contextual fear memory acquisition and extinction is a process that depends on several brain regions, mainly the hippocampus, ventral medial prefrontal cortex (vmPFC), and the amygdala [2]. PTSD involves a hyperactive amygdala [3] and reduced vmPFC activity [4]. Also, individuals with PTSD have in average smaller volumes of regions of the prefrontal cortex, right temporal lobe, corpus callosum, and hippocampus [5].

A recent study investigated contextual fear extinction in persons that experienced combat with and without PTSD [1]. Conditioning was induced in the participants using a light paired with a mild electric shock, and after subjected to fear extinction in a different context. Fear extinction and renewal were assessed by measuring skin conductance response, and brain analyzed using functional magnetic resonance imaging [1]. The results show that participants with PTSD presented impaired extinction recall, expressed as increased skin

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conductance and heightened amygdala activity in the extinction context. They also showed impaired fear renewal in the danger context, and lower amygdala and vmPFC activity compared with controls. PTSD patients did not display enhanced fear expression but instead showed a reduced capability to use contextual information to restrain fear expression when compared to controls [1]. They concluded that contextual fear memory dysfunction is one of the leading problems triggering PTSD pathology [1].

The inability to extinguish fear is often accompanied by depression, a primary risk factor for suicidal behavior, and other medical conditions such as cardiovascular events [6], fibromyalgia, and cancer [7, 8]. For example, the primary depressive disorder is associated with increased risk and mortality rate induced by coronary heart disease [9]. Depressive symptoms in PTSD patients are treated with selective serotonin reuptake inhibitors (SSRIs) [10, 11]. However, less than 60% of patients with PTSD are responsive to available treatments [12]. Thus, the investigation of new therapies against treatment-resistant depression in PTSD is fundamental.

The neural circuits of fear are similar in most of the species of vertebrates [13]. For this reason, for many decades, researchers have used rodent models to model depression, anxiety, and learning and memory dysfunction using stress paradigms such as restraint stress, fear conditioning, and forced swimming [13]. Animal models of stress have been central to the discovery of molecular and cellular mechanisms underlying stress-induced depression as well as the study of the effectiveness of new therapeutic compounds. Using rodent models of stress PTSD, it has been demonstrated that cotinine, an alkaloid derived from tobacco, relieves stress-induced cognitive impairment, depressive-like behavior, and anxiety [14–18].

Cotinine has antidepressant and anxiolytic effects, as well as promotes working memory abilities in subjects subjected to acute or chronic psychological stress. Cotinine has a long plasma half-life (> 19 h), and no clinically significant side effects in humans [19–24]. Furthermore, cotinine has anti-inflammatory properties, overriding the production of cytokines that are under transcriptional nuclear factor-κB (NF-κB) system control such as transforming nerve growth factor (TNF)α, interleukin (IL)-1β, IL-6, IL-12, and IL-23 [25].

Current evidence suggests that cotinine, a metabolite of nicotine, is a positive allosteric modulator (PAM) of the α7 nicotinic acetylcholine (ACh) receptor (α7 nAChR), increasing its stability and response to its agonist ACh [26, 27]. At the cellular level, cotinine increases synaptic density and protects astrocytes in the hippocampus and frontal cortex of mice subjected to restraint stress [28].

A healthy synaptic function in the hippocampus and frontal cortex is required to maintain mood stability [29–32]. Synaptic function and plasticity depend on a delicate balance between phosphorylation and dephosphorylation of synaptic factors by protein kinases whose activity is influenced by neurotransmitter receptors such as the nAChRs [33]. Cotinine activates the extracellular signal-regulated kinases (ERK)s and the Akt/glycogen synthase kinase 3 beta (GSK3β) pathway and promotes the expression of the synaptic proteins such as postsynaptic density protein 95 (PSD95) and synaptophysin in the brain of rodents subjected to stress [28]. Furthermore, cotinine also activates these pathways in the brain of rodents suffering from neurodegenerative conditions [34–36]. In contextual fear memory, after re-exposure to the training context, the retrieval of the memory triggers two conflicting processes: reconsolidation or extinction [37]. Repeated exposure in the absence of the original noxious stimuli induces extinction. During fear memory consolidation and extinction, there is activation of protein kinases such as ERKs [18, 38] and transcription factors. Some of the transcription factors involved in fear memory processes are the activator protein 1 (AP-1), the cAMP response element-binding protein (CREB) [39], the nuclear factor of activated T cells (NFAT) [40], the NF-κB [40–42], and the zinc finger-inducing factor 268 [43, 44].

Synaptic function during plasticity processes involved in memory acquisition and extinction also depends on the timely dephosphorylation of signaling factors by protein phosphatases such as the Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase calcineurin [45–47]. Calcineurin exists as a heterodimer consisting of a catalytic subunit (CaA) and a regulatory subunit (CaB) [48]. Calcineurin A has two isoforms: CaAα that is encoded by two different genes, PPP3CA and PPP3CB, and CaAβ encoded by PPP3CC [49]. One of the main downstream factors of calcineurin is NFAT whose activity is controlled in cortical or hippocampal neurons by calcium levels and the brain-derived neurotrophic factor (BDNF) [41, 50]. Intraneuronal calcium levels are tightly controlled by numerous factors such as L-type voltage-gated calcium channels, calcium pumps, ryanodine receptors in the sarcoplasmic reticulum, and ligand-gated receptors such as the *N*-methyl-D-aspartate receptor (NMDAR) and the α7nAChRs. Changes in calcium levels will then stimulate protein kinases such as the Ca^{2+}/calmodulin-dependent protein kinases (CaMK) and phosphatases such as calcineurin. Calcineurin is expressed in neurons and astrocytes and enriched in the hippocampus and the striatum regions of the brain. A critical study performed using fear conditioning in mice showed that the inhibition of both calcineurin and NFAT in the hippocampus impaired extinction without affecting the reconsolidation of memory. However, NF-κB inhibition diminished memory reconsolidation and augmented its extinction [41]. The calcineurin/ NFAT pathway regulates neurotrophin- and netrin-dependent gene expression during axon outgrowth [51] [52]. NFAT dephosphorylation by calcineurin permits

its translocation into the nucleus. In hippocampal neurons, NFAT activity is stopped by phosphorylation and translocation back to the cytosol induced by GSK3 [53, 54]. The activation of calcineurin is stimulated by a brain-derived neurotrophic factor (BDNF) as well as may require Ca^{2+} influx through VGCCs [52, 55]. Current evidence suggests that calcineurin is involved in the processing of emotional information such as fear and mediating the effect of antidepressants [56].

The activation of cAMP response element binding protein (CREB) by phosphorylation is required for long-term fear memory [57–59]. Calcineurin can terminate CREB activity by dephosphorylation or activation of the downstream protein phosphatase 1 (PP1) [60].

It is thought that oxidative stress in the brain is a crucial factor triggering synaptic plasticity deficits and neuroinflammation in treatment-resistant depression induced by stress [61]. It is known that the imbalance of the immune system induced by stress leads to cerebral neuroinflammation and oxidative stress in subjects with mood disorders [62–67].

The consumption of seafood correlates with lower levels of depression and better cognitive abilities. These benefits have been attributed to their content of antioxidants and anti-inflammatory components [68–71]. Thus, during the search for a neuroprotective additive for an intranasal cotinine formulation, krill oil appeared as an excellent alternative because of its antioxidant and anti-inflammatory properties [72]. Krill oil is extracted from the Antarctic microcrustacean *Euphausia superba* and is a rich source of phospholipids, astaxanthin, and (n-3)/polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [73, 74]. Studies in baboon neonates have shown that absorption of phospholipid-attached PUFAs into the brain is better than the one attained by using triacylglycerol molecules [75]. Numerous reports revealed some beneficial effects of krill oil on depression, anxiety, and cognitive abilities [68, 69, 72–74, 76]. The effectiveness of krill oil seems to rely on the composition of the krill oil preparation as a recent meta-analysis supported the improvement of primary depression by n-3 PUFAs only if the supplements contained more than 60% of EPA [77].

IN delivery of cotinine plus an antioxidant was assayed to improve its therapeutic efficacy and decrease both the amount required for treatment and potential difficulties derived from crossing the blood-brain barrier [78, 79]. However, to bypass the blood-brain barrier is not the only advantage of using intranasal drug delivery. In fact, this form of administration could augment drug efficacy. In fact, intranasal administration represents an attractive alternative to parenteral and oral routes since, also, to be non-invasive, it also avoids gastrointestinal and hepatic first-pass metabolism and prevent its modification and toxicity, thus increasing its therapeutic index [80]. More importantly, for antidepressant drugs, the fast onset of action and rapid delivery to the brain by intranasal route may permit more successful management of emergency mental situations [81].

Distinct types of therapeutics have successfully been intranasally delivered to the brain including cytokines such as interferon β-1b the neuropeptides [82], neurtrophic factors [78], insulin [83, 84], dexamethasone [85], oxytocin [86, 87], cholinesterase inhibitors [88], and manganese [89].

Some studies showed evidence that the effectiveness of IN delivery depends on the physicochemical properties of the therapeutics including molecular weight and lipophilicity; however, there is some discussion of the validity of these assumptions. Numerous small molecule drugs such as procaine, tetracaine, bupivacaine, and lidocaine are efficiently delivered into the CNS via direct nose-to-brain transport [90]. In rodents, small molecules can be administered IN with good bioavailability (AUC intranasal/AUC intra-arterial) of 43 to 100% [90]. In this study, it was investigated the effect of IN cotinine, krill oil, cotinine plus krill oil, or oral sertraline on fear extinction and depressive-like behavior, in mice subjected to fear conditioning. We compared cotinine to sertraline, a selective serotonin reuptake inhibitor (SSRI) approved for treating PTSD in humans [91, 92]. Sertraline permits to achieve clinical improvement mostly temporal, in a good percentage of patients, but it also fails in many of them, especially the patients subjected to war-related experiences [93, 94]. Also, sertraline has a delayed therapeutic effect. Also, due to the significant role of calcineurin in the extinction of fear, we investigated the effect of cotinine on the expression of calcineurin in the hippocampus of mice subjected to fear conditioning and extinction. The effect of cotinine on calcineurin expression is discussed regarding cotinine's effects on depressive and fear memory extinction.

Materials and Methods

Drugs

Cotinine (5S-1-methyl-5-(3-pyridyl) pyrrolidine-2-ona) was obtained from Sigma-Aldrich (Saint Louis, MO). Sertraline hydrochloride (1S,4S)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-*N*-methyl-1-naphthalenamine hydrochloride) was obtained from Sigma-Aldrich. Three hundred milligram soft gels capsules of krill oil omega-3 were purchased from Walgreens (Superba, USA). Capsules contained in 300 mg krill oil (90 mg omega-3 fatty acids, 50 mg EPA, 24 mg DHA (docosahexaenoic acid), 130 mg phospholipids). The manufacturers do not report the astaxanthin content in the soft gels.

Animals

Mice C57BL/6 were obtained from the University of Chile (Santiago, Chile) and maintained on a 12-h light-dark cycle with ad libitum access to food and water.

Mice were maintained grouped (two to three mice by cage) in a controlled environment with average temperatures between 21 and 23 °C and 50–70% humidity. Mice were kept according to the mandate of the Guide of Animal Care and Use of Laboratory Animals of the National Institute of Health (NIH publication 80-23/96). All efforts were made to minimize animal suffering and to reduce the number of animals used. Protocols were performed with the approval of the institutional animal care and use committees of the University of San Sebastián, Chile.

Experimental Design

This study investigated the effect of intranasal cotinine formulations, krill oil, and oral sertraline on depressive-like behavior, fear consolidation, and extinction as well as the expression of calcineurin A in the hippocampus of mice (Fig. 1).

Drug Treatments

Three-month-old mice ($n = 5$–6/condition) were weighed and assigned to treatment groups. Mice were treated with vehicle or drugs, starting 2 h after fear conditioning and daily after behavioral testing until euthanasia. Mice received daily treatments with (1) PBS (phosphate-buffered saline, pH 7.4) via intranasal; (2) cotinine (10 mg/ml) dissolved in PBS via intranasal (IN Cot, 24 μl); (3) krill oil dissolved in PBS, via intranasal (48 mg/ml, 24 μl); (4) cotinine (10 mg/ml) plus krill oil dissolved in PBS, via intranasal (48 mg/ml, 24 μl); and (5) sertraline, via oral in PBS (3 mg/kg, 50 μl). The dose of sertraline was chosen to be equivalent to a 200 mg/day in humans. The dose of cotinine was 10 times lower than the dose of oral cotinine promoting fear extinction in C57BL/6 mice.

Intranasal Delivery

The intranasal delivery was performed according to the protocol of awaken intranasal drug delivery [95]. First, mice were subjected to simulated delivery for 1 week before treatments to reduce the stress due to the procedure.

For intranasal delivery, mice were hand-restrained, placed in a supine position, and given two 12 μl drops of cotinine solutions, or PBS, into both nares consecutively. Mice were given an extra 12 μl treatment drop if the subject forcibly ejected or sneezed out the solution. Mice were held supine for 5–10 s after delivery to ensure that all fluid was inhaled. These volumes have shown to deliver drugs mostly to the brain without passage to the pulmonary regions [95].

Behavioral Analysis

Mice were conditioned and subjected to fear retention test, and extinction trials until extinction was attained. After extinction, mice were tested for depressive-like behavior, using the forced swim test.

Fear Conditioning

Contextual fear conditioning was performed as described [18]. The conditioning chamber used (33 cm × 20 cm × 22 cm) is surrounded by a sound-attenuating box with a camera connected to freeze frame software (MED Associates Inc.) and equipped to provide a background white noise (72 dB). The conditioning chamber contains in one side a speaker and in the opposite side has a 24 V light and a 36-bar insulated shock grid floor. To perform this test, each mouse was placed in the conditioning chamber for 2 min before the onset of a discrete tone (a sound that will last 30 s at 2800 Hz and 85 dB). In the last 2 s of this tone, mice received a foot shock of 1 mA, kept in the conditioning chamber for 2 min, and

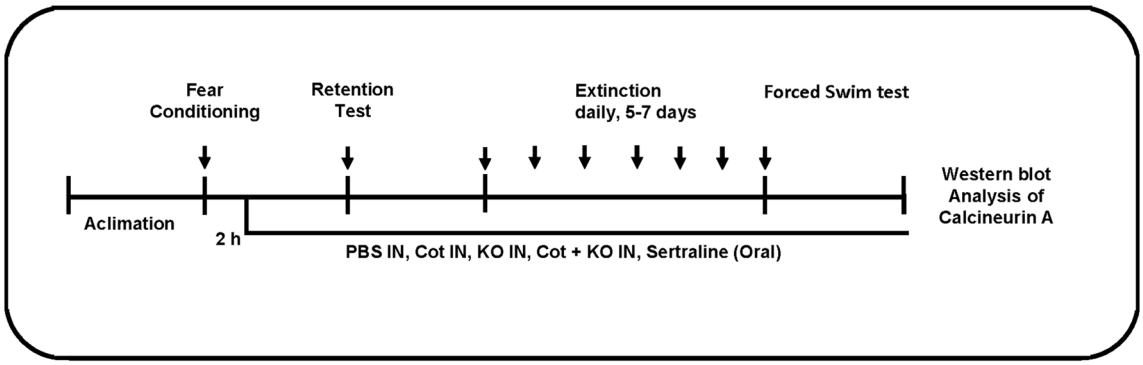

Fig. 1 Diagram representing the experimental design. Male mice ($n = 5$–8/condition) were housed and habituated to their cages before FC. After this period, mice were fear conditioned, treated, behaviorally tested for fear retention and extinction, and depressive-like behavior, and euthanized. Calcineurin analysis was then performed in hippocampal extracts of mice by Western blot

returned to their home cages. Between trials, the chamber was sanitized with 70% ethanol and dried. Freezing behavior was defined as the absence of all movements except the one needed for breathing was assessed using the FreezeView Software (MED Associates Inc.).

Fear Retention and Extinction Tests

Fear retention and extinction experiments were performed as described [18], using the same cohorts of mice and reproduced in two separate experiments. To assess fear retention, mice underwent re-exposure to the conditioning chamber in the absence of an unconditioned stimulus (shock or auditory cues) for 3 min in daily extinction trials. Freezing behavior was measured using the ANY-maze® software (Stoelting CO, USA). The extinction trials were continued until the decrease in freezing behavior reached a stable level.

Forced Swim Test

The forced swim test is broadly used to assess depressive-like behavior [96, 97]. Each mouse was placed in a transparent cylinder (60 cm × 20 cm) filled with water at 25 °C for 5 min. Two investigators blind to all treatment levels scored immobility during the complete time of the assay. A mouse was considered immobile when it remained floating motionless or moved only that which was necessary to keep its head above the water. The time immobile is considered a measure of depressive-like behavior in rodents, and antidepressants decrease the time of immobility in this test [96, 97].

Western Blot Analysis

After the behavioral testing, mice from all treatment groups were euthanized via cervical dislocation by a well-trained investigator. Brain regions of interest were dissected and stored at − 20 °C for protein analyses. Each brain was divided into two parts: left and right hemispheres. The frontal cortex and hippocampus were dissected from left hemisphere on ice and disrupted by sonication in cold cell lysis buffer containing phosphatase and protein inhibitors (Cell Signaling Technology, Danver, MA, USA) and 1 mM PMSF (Sigma-Aldrich Corporation, St. Louis, MO, USA). After sonication, brain extracts were incubated on ice for 30 min and centrifuged at 20,000× *g* for 30 min at 4 °C. The protein concentration of the supernatants was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by gradient 4–20% SDS-PAGE then transferred to nitrocellulose membranes (BA83 0.2 μm; Bio-Rad). The membranes were blocked in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) containing 10% dry skim milk for 45 min. Membranes were incubated with primary antibodies in TBST overnight at 4 °C, and with secondary antibodies for 1–3 h at RT in blocking buffer. A rabbit polyclonal antibody directed against calcineurin (PP2B) was obtained from Cell Signaling Technology. A monoclonal antibody directed against total Akt (Cell Signaling) was used to control protein sample loading and transfer efficiency. Membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies (Bio-Rad) for 1 h at RT and washed with TBST and TBS, and images were acquired using My ECL imaging system and analyzed using the NIH ImageJ software.

Statistical Analysis

All values expressed as mean ± standard error of the mean. The behavioral and immunoreactivity differences between sample and treatment groups were determined by one-way or two-way analysis of variance (ANOVA) with post hoc Tukey analysis. $p < 0.05$ was considered as statistically significant. All statistical analyses were performed with the software GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effect of the Combination of Cotinine and Krill Oil on Fear Retention After Fear Conditioning

Fear conditioning (FC) was used to assess the effect of post-treatment with IN cotinine on fear memory acquisition and consolidation in mice. Each mouse was conditioned, and 2 h later started on treatments. One-way ANOVA analysis revealed a significant effect of treatments on fear retention ($F(4, 22) = 4.964$, $p = 0.005$). A multiple comparison post hoc Tukey's analysis revealed that compared to non-stressed (NS) mice, mice treated with intranasal cotinine ($p < 0.05$) and cotinine plus krill oil ($p < 0.01$) showed a significant decrease in the fear reaction in the retention test. On the contrary, no significant effects of krill oil alone or oral sertraline were observed (Fig. 2).

Cotinine and Cotinine Plus Krill Oil Enhanced Contextual Fear Extinction

The effect of cotinine and krill oil on the extinction of contextual fear memory was assessed by measuring freezing behavior during the daily extinction trials; all groups of mice showed a decrease in freezing that reached a steady decrease by day 5. However, a repeated measure ANOVA throughout the 5 days of extinction revealed a significant difference induced by treatments ($F(1762, 7046) = 6001$, $p = 0.0324$) and days ($F(4, 16) = 42{,}19$, $p < 0.0001$) on the freezing behavior. Cotinine- and cotinine plus krill oil-treated mice showed a

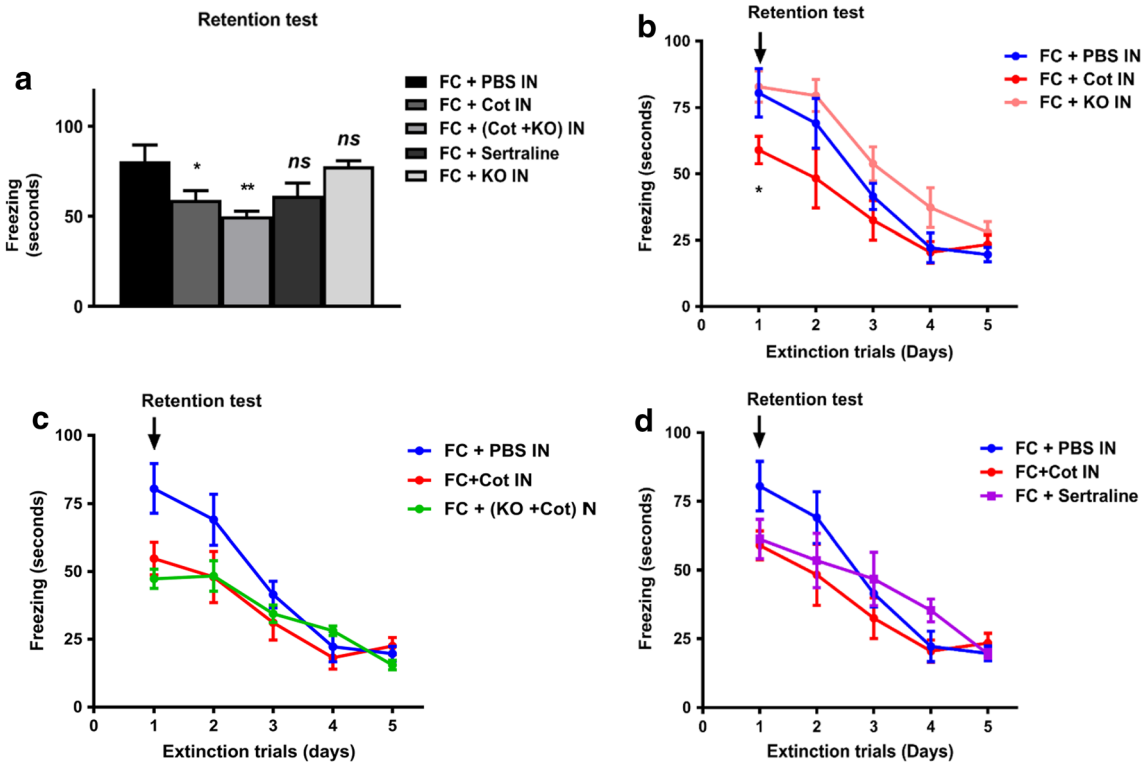

Fig. 2 Effect of early posttreatment with cotinine, sertraline, and krill oil on the retention and extinction of fear memory. Two hours after fear conditioning (FC), mice ($n = 5–8$ mice/group) received intranasal (IN) PBS, krill oil (KO) IN, cotinine (Cot) IN, or Cot + KO IN, oral sertraline (2 mg/day). Next day after, mice were tested for contextual fear memory (retention test) and subjected to daily trials of fear extinction until a minimun and stable freezing behavior was reached. The graphs depict the freezing behavior during the retention test (**a**) and during the extinction trials in mice treated with PBS IN, Cot IN, KO IN (**b**); PBS IN, Cot IN, Cot + KO (**c**); and PBS IN, Cot IN, oral sertraline (**d**). Data was analyzed using one-way ANOVA and Tukey post hoc test. *ns* non-significant change; $*p < 0.05$; $**p < 0.01$

faster extinction of fear, but they reached a maximal decrease at the same time than mice treated with PBS, on day 4 (Fig. 3a, b). Separately, mice treated with sertraline or krill oil alone showed an overall slower extinction of fear than controls, reaching a decrease that was comparable to control PBS-treated mice only on day 5 (Fig. 3c).

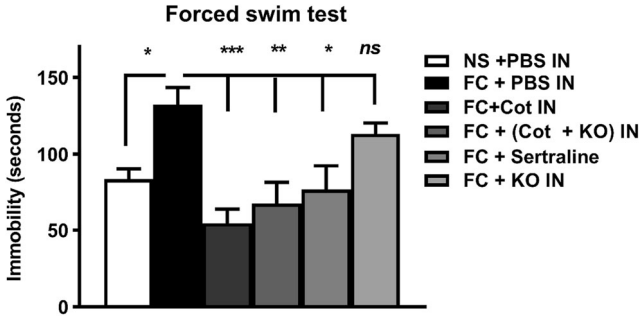

Fig. 3 Effect of cotinine and krill oil on depressive-like behavior in the forced swim tests. Two hours after fear conditioning (FC), mice ($n = 5–8$ mice/group) received oral sertraline (2 mg/day), intranasal (IN) krill oil (KO), IN cotinine (Cot) (24 μl, 10 mg/ml), or IN Cot plus KO and subjected to fear extinction. The graphs depict the effect of treatments on freezing behavior as measure of depressive-like behavior. Data was analyzed using one-way ANOVA. *ns* non-significant change; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

Effect of Cotinine, Sertraline, and Krill Oil on Depressive-Like Behavior in Conditioned Mice

The data revealed that the conditioned mice subjected to fear extinction presented a significant decrease. The analysis of depressive-like behavior data in the forced swim test revealed a significant difference between treatment groups (one-way ANOVA, $F(5, 38) = 6.32$, $p = 0.0002$). Mice subjected to FC with a single shock showed higher levels of depressive-like behavior than NS mice treated with PBS ($p < 0.01$). FC mice posttreated with intranasal cotinine and sertraline showed a significant decrease in depressive-like behavior ($p < 0.05$) expressed as a higher immobility times in the forced swim test. A Tukey's post hoc analysis showed that intranasal cotinine ($p < 0.001$), the combination of cotinine plus krill oil ($p < 0.005$), and sertraline ($p < 0.05$) significantly decreased depressive-like behavior expressed as a decrease of immobility values in the forced swim test (Fig. 4). Krill oil showed an antidepressant effect, but this did not show significance.

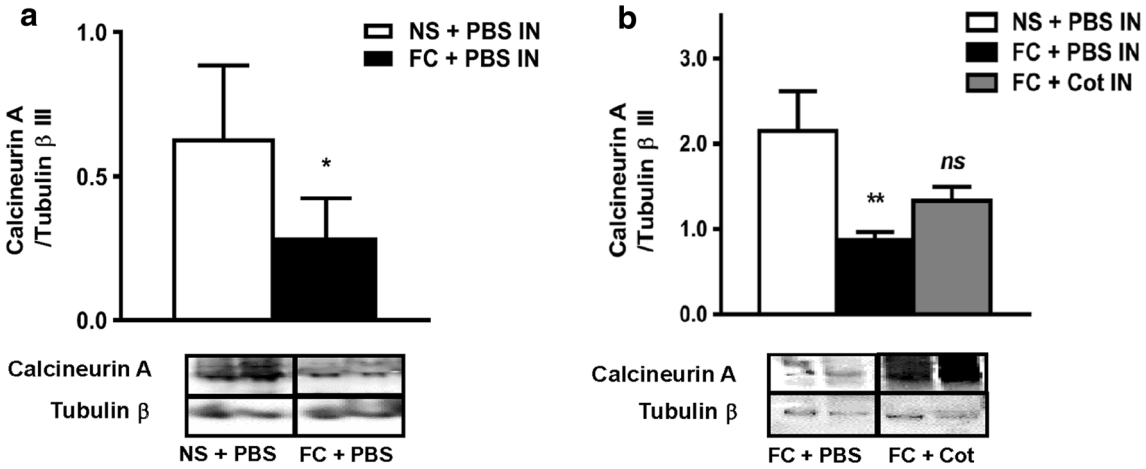

Fig. 4 Cotinine increased calcineurin A in the conditioned mice after fear extinction. The hippocampal expression of calcineurin A (CaA) was analyzed by Western blot in the mice after fear conditioning (FC) and extinction (FE). The graphs represent the expression of calcineurin in the hippocampus of control non-exposed to stress (NS) and conditioned (FC) mice treated with PBS (vehicle) (**a**) and NS conditioned mice treated with PBS, plus cotinine (Cot) (**b**). *ns* non-significant change; $*p < 0.01$; $**p < 0.05$

Effect of Cotinine on Calcineurin A in the Hippocampus of Conditioned Mice

Previous studies showed that calcineurin A is involved in the neuronal changes associated with fear extinction [40, 98, 99] and that antidepressants increase its expression [100] (Fig. 5). Thus, based on the antidepressant activity of cotinine, the effect of intranasal cotinine on the hippocampal expression of calcineurin A in NS and conditioned mice was investigated. The conditioned mice showed a significant decrease in the levels of calcineurin A (Student's t test, $t = 2597$ df $= 7$, $p = 0.036$) when compared to NS mice in the hippocampus (60% decrease) (Fig. 4a). However, significant changes in the expression of calcineurin A between treatment groups were found (one-way ANOVA, $F(2, 13) = 6, 26$, $p = 0.013$). Mice posttreated with intranasal cotinine showed calcineurin A levels in the hippocampus significantly different from PBS-treated conditioned mice ($p < 0.001$) (Fig. 4b). No significant differences were observed between PBS-treated conditioned mice and krill oil or krill oil plus cotinine-treated mice ($p > 0.05$).

Discussion

An optimal drug to prevent or treat PTSD may target the main aspects of the disease in a rapid, inexpensive, and targeted manner. Current evidence showed beneficial effects of cotinine on working memory, anxiety, depression, and the extinction of fear in mouse models of PTSD [14, 18, 101, 102]. In here, it was investigated the effect of intranasal cotinine alone

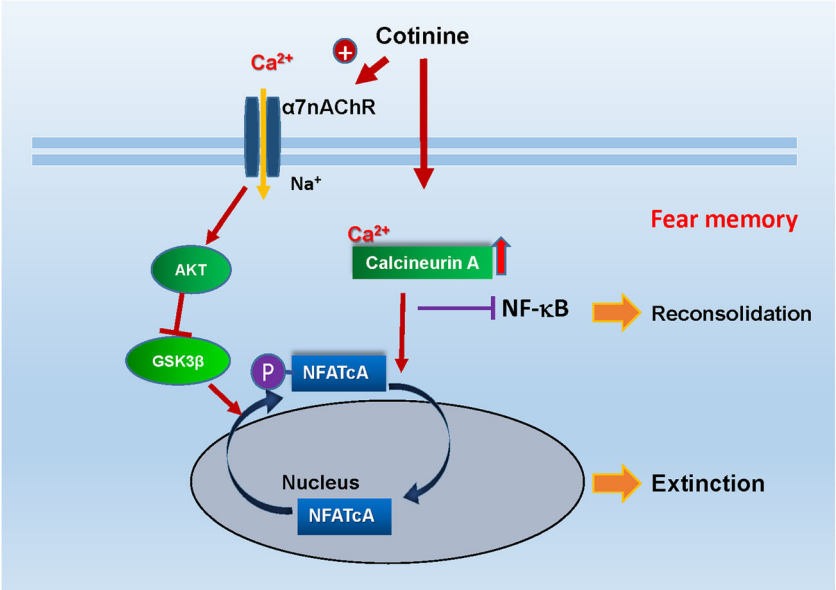

Fig. 5 Potential effects of cotinine on calcineurin A activity during extinction. The diagram despict the activation of cotinine enhancing the activation of the α7nAChR and the consequent activation of Akt and calcineurin and the inactivation of GSK3β and NF-κB. Calpain by dephosphorylation of NFAT and inhibition of GSK3β will stimulate the expression of genes involved in extinction and will inhibit transcription factors involved in consolidation of fear memory such as NF-κB

or combined with krill oil on depressive behavior and the consolidation and extinction of contextual fear memory in mice. Intranasal cotinine preparations when administered 2 h after conditioning efficiently reduced the consolidation or retrieval of contextual fear memory, enhanced the extinction of the fear responses, and diminished depressive-like behavior in mice. The mix cotinine plus krill oil was superior to cotinine alone in preventing the consolidation of fear memory and in diminishing depressive/like behavior after fear conditioning. Intranasal krill oil alone delayed the extinction of the fear memory. When the effect of conditioning and extinction on calcineurin A expression was determined, it was found that after extinction, there was a reduced expression of calcineurin A in the hippocampus of conditioned mice treated with PBS, while cotinine increased its levels.

No previous studies have investigated the effect of intranasal cotinine preparations on the extinction of contextual memory. The fact that cotinine was effective by intranasal delivery, using a technique probed to deliver drugs to the brain mainly (> 90%), suggests that cotinine and not a liver-derived metabolite of this alkaloid is responsible for its beneficial effects. On the other hand, IN delivery probed to be effective at doses of cotinine 10 times lower than previously reported doses enhancing fear extinction [18].

Overall, cotinine preparations were superior to sertraline in diminishing fear responses, while having similar effect diminishing the consolidation of fear memory and the ensuing depressive-like behavior in mice. In rodents, sertraline is [103] more effective in females than its male counterparts [104]. Only a few studies have investigated the effects of antidepressants on the extinction of aversive memories, and the conclusions derived from them are no very consistent [105, 106]. It has been reported that in male rodents, sertraline did not diminish anxiety. Furthermore, during extinction, sertraline increased the time spent defensive that declined during the consecutive sessions [104]. The present study shows that cotinine plus krill oil is effective in decreasing fear memory consolidation and diminishing depressive-like behavior. To our actual knowledge, the only probed targets of cotinine are the nAChRs, whose stimulation or stabilization is thought to enhance synaptic plasticity, to decrease neuronal and astrocyte damage, and to reduce neuroinflammation [15, 27]. Interestingly, krill oil contains phosphatidylcholine that may be used to synthesize acetylcholine, as a ligand to bind nAChRs [74]. This effect may further potentiate the beneficial effects of cotinine stabilizing the receptor in the plasma membrane and positively modulating its function.

Previous studies showed evidence suggesting that krill oil improves cognitive abilities, decreases depressive-like behavior, and reduces inflammation in rodents [68, 71, 76]. One of these studies found increased brain cell generation in the dentate gyrus of the hippocampal formation, and a decrease of reactive oxygen species in the cerebral cortex and hippocampus of krill oil-treated rats [68]. Furthermore, rats subjected to forced swim stress and treated with krill oil or imipramine showed reduced immobility times in the forced swim test and an improvement in memory functions than control animals [68]. This evidence suggests an improvement in cognitive abilities and mood induced by krill oil supported by a decrease in oxidative stress. Recent studies have shown that components of the krill oil, n-3 long-chain polyunsaturated fatty acid (PUFA) and n-6 PUFA (3:6) ratio influences fear memory. They examined several dietary 3:6 ratios on fear memory in mice subjected to contextual fear conditioning and showed that fear memory expression correlated negatively with dietary, serum, and brain 3:6 ratios in mice. A pharmacodynamic analysis in mice fed a high 3:6 ratio diet revealed that the PUFA acted through the CB1 receptor (CB1R) and increased short-term synaptic plasticity in the pyramidal neurons of the BLA. The authors suggest that the ratio n-3 to n-6 PUFA regulates fear memory via cannabinoid CB1 receptors (Yamada 2014). PUFA seems to control the levels of endogenous agonist of CB receptors (Watkins 2010). In the present study, a positive effect of krill oil alone in diminishing fear memory consolidation or enhancing its extinction was not found. On the contrary, a delay in the extinction of contextual fear memory response was observed. The different outcome may be related to the length of the administration and the sex of the subjects. In this study, mice were treated short time after the conditioning to investigate the potential of intranasal krill oil as a standalone treatment or adjuvant treatment for cotinine in preventing the consolidation of fear memory and its extinction. The superior effect of cotinine in combination with krill oil attained in mice is encouraging and suggests a similar enhancement of the beneficial effects also in humans. The synergic effect of cotinine plus krill oil, in the absence of an effect of krill oil alone, suggests that the potentiation of cotinine effects by krill oil may be responsible for the improved effect of the mix.

Calcineurin has been implicated in the consolidation and stability of newly acquired memories [107]. Previous reports stated that psychological stress inhibits the expression of calcineurin A and that it can be later restored by antidepressants [100]. This evidence agrees with our results showing that fear conditioning decreased the expression of calcineurin A in the hippocampus and that the antidepressant effect of intranasal calcineurin treatment corrected this decrease. Also, it has been shown that the overexpression of calcineurin in the forebrain decreased the rate of learning in fear conditioning tasks [45, 46]. Coincidently, we found that cotinine preparations that increased calcineurin A expression inhibited the consolidation of contextual fear memory. Furthermore, it has been defined an essential role of calcineurin in memory extinction or behaviors requiring behavioral inhibition [107]. The authors suggested that calcineurin is involved in behavioral flexibility [107].

Overall, the results suggest that short-term treatment with intranasal cotinine plus krill oil is superior to sertraline and krill oil alone in enhancing fear extinction. Despite krill oil plus cotinine is only slightly superior to cotinine alone in decreasing fear responses, the use of the mix with krill oil has the added advantage that krill oil has beneficial effects over the vascular health. Thus, cotinine plus krill oil delivered intranasally represents a right combination for the treatment of people with PTSD that have a higher incidence of vascular diseases. Further clinical studies would be required to fully confirm the therapeutic value of intranasal cotinine alone and combined with krill oil for facilitating the recovery of people with PTSD. The evidence shows that cotinine intranasal alone or in combination with krill oil facilitates the extinction of contextual fear memory and diminish depressive behavior at a dose 10 times lower that the previously active oral dose of cotinine in mice. The pro-cholinergic, antioxidant, and anti-inflammatory effects of both compounds may explain their synergic positive effects on depression. The effect of cotinine on calcineurin A seems to be another critical mechanism of action of cotinine against PTSD pathology, but the topic requires further investigation to demonstrate a direct causal relationship with cotinine beneficial effects. Overall, this preclinical evidence supports the clinical investigation of intranasal cotinine plus krill oil to reduce depressive symptoms derived from recurrent associative trauma memories in PTSD patients.

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Compliance with Ethical Standards

Conflict of Interest VE is the inventor of the approved patent US 20100104504 (August 2017).

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Artículo N°4

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Cotinine: A Therapy for Memory Extinction in Post-traumatic Stress Disorder

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Abstract
Post-traumatic stress disorder (PTSD) is a mental disorder that may develop after exposure to exceptionally threatening or unescapable horrifying events. Actual therapies fail to alleviate the emotional suffering and cognitive impairment associated with this disorder, mostly because they are ineffective in treating the failure to extinguish trauma memories in a great percentage of those affected. In this review, current behavioral, cellular, and molecular evidence supporting the use of cotinine for treating PTSD are reviewed. The role of the positive modulation by cotinine of the nicotinic acetylcholine receptors (nAChRs) and their downstream effectors, the protection of astroglia, and the inhibition of microglia in the PTSD brain are also discussed.

Keywords Post-traumatic stress disorder · Cotinine · Fear · Inflammation · Nicotinic receptor · Extinction

Abbreviations

AD	Alzheimer's disease
Akt	Protein kinase B
AMY	Amygdala
BDNF	Brain-derived neurotrophic factor
CaMKK	Calmodulin-dependent protein kinase (CaM kinase)
CAPS	Clinician-administered PTSD scale
CR	Conditioned response
CREB	Cyclic AMP response element-binding protein
DSM	Diagnostic statistical manual of mental disorders
FC	Fear conditioning
fMRI	Functional magnetic resonance imaging or functional MRI
GABA	Gamma-aminobutyric acid
ICN	Intercalated nuclei
GSK3	Glycogen synthase kinase
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa-light-chain enhancer of activated B cells
nAChRs	Nicotinic acetylcholine receptors
NT	Neurotrophin
PDK-1	PI-dependent kinase 1
PFC	Prefrontal cortex
PTSD	Post-traumatic stress disorder
VEGF	Vascular and endothelial growth factor

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Introduction

Post-traumatic stress disorder (PTSD) can occur after a single traumatic event or from prolonged exposure to trauma [1–4]. Only four decades ago, PTSD was recognized as a disorder

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with specific symptoms that could be reliably diagnosed and was added to the American Psychiatric Association's Diagnostic Statistical Manual of Mental Disorders (DSM). Recently, the DSM 5 have introduced changes of the disease classification to distinguish it from other anxiety disorders.

Stress exposure initiates physiological and psychological responses that prepare the organism to cope with the environmental change. Fear elicited by an imminent danger of death triggers in split seconds the "fight-or-flight" response that consists in neuronal, endocrine, and behavioral responses, which are directed to avoid or confront dangerous situations. These healthy physiological reactions prepare individuals to escape from an imminent danger, but extinguish after the event; however, in people with PTSD, this fear reaction continues to appear in absence of danger due to a failure in the fear memory extinction process [2, 5–8].

There have been different conceptual models or criterions proposed to diagnose PTSD according to clusters of symptoms (from four to seven factors). Some of these clusters are common for all of them such as re-experiencing symptoms (intrusive thoughts, nightmares, flashbacks, and emotional and cue reactivity); and avoidance symptoms (avoidance of thoughts, avoidance of remainders); however, they differ in the classification of other clusters. For example, the DSM 5 considers that many symptoms (trauma-related amnesia, negative beliefs, blame of self or others, negative trauma-related emotions, loss of interest, detachment, restricted affect) are alterations in cognition and mood in comparison with other models that classify them as dysphoria or anhedonia clusters [9]. Similarly, alterations in arousal and reactivity in DSM 5 criteria (irritability/anger, self-destructive/reckless behavior, hypervigilance, exaggerated startle response, difficulty concentrating, and sleep disturbances) are considered in other models as externalizing behaviors (irritability/anger, self-destructive and reckless behavior); anxious arousal (hypervigilance, exaggerated startle response); or dysphoric arousal (difficulty concentrating and sleep disturbance) [10]. In addition, according to the DSM 5 criteria for PTSD, the diagnosis requires that a stressful event must precede the appearance of symptoms, including intrusive thoughts, increased evasion, cognitive impairment, increased emotional reactivity, and arousal. These symptoms must be persistently displayed over time and significantly alter the social functioning of the affected individual [10].

The global prevalence of PTSD fluctuates between 3 and 7% of the adult population at any point of time. The disorder can develop at any age including childhood and affects about 7.7 million of adults only in North America each year. According to some studies, the countries with the highest prevalence of PTSD, which is the proportion of cases in the population at the time of this study, were Netherlands, UK, France, and Germany, while countries with the lowest prevalence of PTSD were Spain and Switzerland [11].

The incidence of PTSD changes according to the type of stressor [12, 13], sex [14, 15], age [16] genetic background [17, 18], and race [19], among other factors. Recent studies have estimated that about 30 to 50% of the general population exposed to natural disasters suffers some psychological disturbance in the months after the traumatic event [16, 20]. These mental disturbances manifest as subclinical distress, acute stress, and maladaptive disorders such as PTSD, depression, anxiety, alcohol, and drug abuse [16]. Meta-analysis study of 46 clinical reports containing data for 76,101 earthquake survivors showed that 17,706 met the criteria for the diagnosis of PTSD with a combined incidence of PTSD after earthquakes of approximately 24% [21]. The incidence of PTSD is also determined by cultural factors, type of trauma, and previous mental state before experiencing trauma. For example, an epidemiological study of trauma revealed that 3 years before the earthquake in 2006, 41.7% of men and 33.2% of women had been exposed to trauma in Chile. At that time, the prevalence of PTSD was calculated in 4.4% [22]. However, recent seismic events, such as the massive earthquake that hit much of the Chilean territory, further increased these percentages and the incidence of PTSD in the Chilean population [23]. Being PTSD symptoms dramatically increased, mainly among residents of areas heavily stroked by intense earthquakes in Chile, compared with people from other sectors [22]. Importantly, this effect was more accentuated in women with previous diagnosis of depression [24]. On the other hand, participation in war combat situations is another stressor that can lead to PTSD. Soldiers are exposed to dangers, such as loss of life, injury, and the death of comrades, as well as to harsh physical conditions, such as extreme weather, lack of sleep, lack of food, isolation, loneliness, and loss of family support. For these reasons, PTSD is a major physical and mental health problem for military personnel and civilians exposed to war events [25–29]. In fact, the incidence of PTSD is higher in subjects exposed to combat (10–40%) than in the civilian population (6–7%) [30, 31].

A meta-analysis of the prevalence of PTSD in service personnel revealed higher prevalence rates of PTSD among Iraq-returned veterans (12.9%) than personnel deployed to Afghanistan (7.1%). Similarly, a study performed in combat deployed personnel from Canada, USA, and UK found a higher prevalence of PTSD in serving personnel from the army, navy, or marines (12.4%) than other services (4.9%) [29].

Potential Neuronal Circuits and Brain Regions Targeted by Cotinine in Mediating Fear Extinction

PTSD is characterized by alterations of cognitive abilities such as attention, learning and memory, planning, and problem solving, thus highlighting the enormous impact of trauma on brain functioning. To understand this impact, many clinical imaging studies have examined differences in brain volume

between patients with PTSD and control subjects. In the brain of PTSD patients, structural changes have been mainly found in the amygdala (AMY), hippocampus, and frontal cortex [32–35]. One of these studies, revealed an inverse correlation between the clinician-administered PTSD scale (CAPS) scores with the volumes of the subgenual cingulate, caudate nucleus, hypothalamus, insula, and left middle temporal gyrus [32]. Furthermore, brain network analysis has been used to assess changes in information flow through anatomical or functional brain networks [36]. To represent brain networks, brain connectivity is determined by investigating the density of white matter tracts between regions, and regional time series correlations during rest or task evoked activity using functional magnetic resonance imaging (fMRI). Recent imaging studies have revealed that PTSD is associated also with changes in brain functional connectivity of these regions involved in emotional regulation and cognition including the cingulate cortex, frontal cortex, hippocampus, and amygdala [34, 35, 37–41]. Alterations in the connectivity between these regions are associated with abnormal fear extinction, emotion, and autonomic hyperarousal [42–44].

Fear conditioning (FC) has been instrumental to investigate the effect of new pharmacological agents for PTSD. The circuit of fear is similar between rodents and humans, thus symptoms of PTSD including fear, depression, anxiety, memory loss, and hyperarousal can be induced by FC in mice and rats [45]. During FC, rodents are exposed to a traumatic unconditioned stimulus paired with a conditioned stimulus (CS), such as a context and/or a sound [45]. When the subject is later confronted with similar or identical CS, a conditioned response (CR) such as freezing occurs [46, 47]. After FC, the extinction of the fear response can be achieved by repetitive exposures to environmental cues that remind the ones present during the traumatic or aversive event [45, 48].

Stress paradigms are linked to a decrease in architectural complexity of neurons and astroglia in rodents. Animal models of PTSD such as restraint stress and FC have revealed that stress induces structural changes in brain cells. In neurons, stress affects the length of apical dendrites and reduces postsynaptic dendritic spines in multiple brain regions. In the hippocampus, chronic stress causes atrophy of apical dendrites in CA1 and CA3 pyramidal cells and a decrease in the density of postsynaptic dendritic spines [49]. Similar changes have been observed in the prefrontal cortex (PFC) [50]. Chronic stress also disrupts neurogenesis in the dentate gyrus [51, 52] and causes hypertrophy of dendritic arbors in the amygdala that parallels a facilitation of aversive learning and heightened fear and anxiety in rodents [53–56].

In humans, similar FC paradigms are used to investigate fear extinction, but in these clinical studies, fear response is usually determined by measuring enhanced skin conductance [57]. Liberzon and colleagues have proposed that a deficit in contextualization represents a key pathological deficit in PTSD and that the inability to adequately process contextual information impairs the ability of people with PTSD to use external and internal contextual cues to discriminate between safe and dangerous situations and to choose an appropriate fear response [58]. Traumatic experiences during childhood provoke long-lasting changes in brain structure by affecting myelination, neurogenesis, and synaptic branching. According to previous studies, these changes result in structural changes of the corpus callosum, altered activity of the frontotemporal region and the cerebellar vermis, and impaired development of the amygdala, hippocampus, and neocortex [59–61]. In addition, it has been found a major reactiveness of the brain regions involved in emotional regulation and the salience network to aversive stimuli in people with a history of trauma. This hyperactivity seems to reflect higher local connectivity between regions within those networks in the brain of these individuals.

One of the distinctive characteristic that differentiate PTSD from several anxiety disorders is the deficit in extinguishing acquired fear memory [57]. The neurobiological bases of fear extinction are not completely understood. Nevertheless, current evidence indicates that extinction requires an efficient communication between the PFC [62], amygdala [63], and hippocampus [64]. Amygdala is required for retrieving and extinguishing fear memories [63]. Extinction of fear requires the activation of GABAergic interneurons in the amygdala by efferent neurons projecting from the infralimbic region of the medial PFC (mPFC) to the intercalated nuclei (ICN) of the amygdala. The ICN consists of a cluster of small GABAergic neurons located in the periphery of the AMY basolateral nuclear complex that sends GABAergic projections to the central nucleus of the amygdala [65]. These projections inhibit the outputs of the amygdala to the hypothalamic and brainstem regions, which are involved in the expression of fear [65–68]. Brain imaging techniques have shown that persons with PTSD have in comparison to control subjects a hyporesponsive mPFC and an overactive amygdala. This evidence suggests that inhibitory loops mediating extinction of fear, which is normally triggered by environmental cues similar to the ones present during trauma, is impaired in subjects with PTSD [58, 69]. These alterations in brain networks are influenced by gender [70–72]. It has been found that regions involved in emotional regulation show a greater response to aversive stimuli in women than their male counterparts [53, 54]. In fact, anatomical and functional differences in the brain have been found between male and females after exposure to trauma during childhood [55]. These differences may explain the differences observed in the PTSD risk between persons of different sex [14, 56–60].

The circuit of fear can be modulated by several neurotransmitters involved in stress responses [73–75]. The neurotransmitters gamma-aminobutyric acid (GABA) and glutamate play a vital role in extinction. GABA and

glutamate, acting at N-methyl-D-aspartate (NMDA) receptors, are involved in GABA-mediated inhibition of the amygdala [75]. Furthermore, these neurotransmitters can be modulated by the cholinergic system [59, 60, 76–78]. The cholinergic system controls synaptic plasticity processes in the mPFC and the hippocampus, both regions involved in contextual fear extinction [58]. Interestingly, the glutamatergic neurons from the mPFC innervating the amygdala contain presynaptic nicotinic acetylcholine receptors (nAChRs), which can facilitate the activation and firing of these excitatory neurons. This activation will stimulate inhibitory GABAergic neurons in the amygdala reducing its activation. Comparable effects of cholinergic neurons can inhibit or activate neurons in the hippocampus that is a key player for contextual fear memory extinction. Thus, it is reasonable to speculate that the activation of the cholinergic neurons in the circuit of fear may facilitate fear extinction [79, 80]. However, it is possible that other receptors are also involved in the potential actions of cotinine stimulating GABA neurons in the amygdala and hippocampus.

Cotinine as an Adjunctive Therapy to Psychotherapeutic Approaches for PTSD

Evidence-based psychotherapeutic approaches include eye movement desensitization and reprocessing, and cognitive behavioral therapies including prolonged exposure and cognitive processing therapy [81]. In persons with PTSD, cognitive behavioral therapies are directed to diminish the abnormal fear response occurring in association with exposure to certain environmental situations that act as reminders of trauma [82]. Several drugs have been used with or without psychotherapy for PTSD symptom alleviation, even though selective serotonin reuptake inhibitors (SSRI) continue to be the more effective ones to date. However, even when they are effective for a percentage of patients (60–70%), the effect is temporary and symptoms reappear after discontinuation of treatment. One of the more successful and broadly used cognitive therapies is the extinction-based prolonged exposure therapy [75, 83, 84]. Prolonged exposure therapy basically consists in facilitating fear extinction by evoking and restructuring traumatic memories in the patients [80, 85]. This therapeutic model is based on the concept that maladaptive cognitive processes can be overcome by "editing" the trauma memory. These therapies are emotionally painful because patients have to relearn a sense of safety by confronting feared representations of traumatic events [86]. Unfortunately, albeit cognitive therapies can be largely effective managing PTSD symptoms, relapse is frequent, even after total extinction was theoretically achieved [87].

Current approved medications for PTSD do not target fear extinction and are not very effective. However, the prospect of using drugs or other interventions to facilitate the psychotherapeutic and other alternative approaches is very appealing. Thus, drugs that permit a maintainable extinction of fear are needed to be used alone or as augmentation drugs to facilitate extinction or consolidate its effects. Today, there is no unmistakable evidence indicating that combining pharmacotherapy plus psychotherapy is more effective than current therapies approaches alone [88]. Absolutely, a deeper understanding of the molecular mechanisms of extinction may facilitate to develop new treatment for patients with PTSD and other mental health disorders showing similar symptoms.

Many studies have shown that physical activity is associated with better psychological well-being, physical health, life satisfaction, and cognitive functioning in various psychiatric disorders [89–98] including PTSD [97, 99, 100]. Studies using therapeutic approaches based on exercise programs have shown significant reduction in symptoms of anxiety and depression in patients with PTSD [101, 102]. However, all these studies have many methodological flaws, such as very small sample sizes, lack of control groups, and inconsistencies in establishing the criteria for the diagnosis of PTSD [103]. Therefore, randomized controlled trials to evaluate its effectiveness in PTSD are still needed [104]. One recent study, including data from 1368 (994 men) participants from 18 to 70 years of age indicated that hyperarousal symptoms were associated with lower physical activity among people with PTSD [95–97]. However, the role of physical activity on the response to therapy among people with PTSD has been not fully investigated and should be assessed by future research. Alternatively, recent studies investigated the effect of deep brain stimulation on fear extinction in rodents. They reported that deep brain stimulation of the amygdala, ventral striatum, hippocampus, and prefrontal cortex facilitated fear extinction and reduced anxiety. Some successful results in one clinical study show encouraging results but new clinical studies are required for this treatment validation [105, 106]. Despite these deficiencies, it is appealing that non-pharmacological interventions may help to enhance the endogenous systems of resilience to stress. It is reasonable to propose that non-pharmacological and/or pharmacological approaches with innocuous natural compounds can facilitate extinction of fear.

Among the natural compounds, alkaloids with precognitive effects can be good therapeutic alternatives. In this regard, we have characterized at the preclinical level, the nicotine-derived alkaloid named cotinine, which positively modulates the cholinergic [107], dopaminergic [108], and serotoninergic systems [109], facilitating brain plasticity and stress resilience, and reducing neuroinflammation [110].

Cotinine Stimulation of Synaptic Plasticity Factors under Stress Conditions

Cotinine can be administered orally, intravenously, intramuscularly, and intranasally without clinically relevant side effects in humans [111–115]. Previous preclinical studies have shown that cotinine improved neuronal survival, and memory abilities in mouse models of Alzheimer's disease (AD) [116–118], autism [119], and schizophrenia [120, 121], as well as restored cognitive abilities after exposure to chemotherapeutic drugs mix in female rats [122]. These studies revealed several molecular targets of cotinine including the protein kinase B (Akt)/glycogen synthase kinase (GSK3) pathway [79, 117–119]. Akt is activated by cotinine, and it has multiple biological functions in the brain, supporting neurogenesis and neural survival and plasticity that are required for learning and memory abilities and mood stability. In fact, Akt has been found to be downregulated in many psychiatric and neurological disorders, supporting the view that its dysregulation can be key in the development of these conditions [123].

Akt is activated by phosphorylation by PI-dependent kinase 1 (PDK-1) at threonine 308 [58], and Ca^{2+}-calmodulin-dependent protein kinase (CaM kinase) kinase (CaMKK) and mammalian target of rapamycin (mTOR) complex 2 at serine 473 [124]. Once active, Akt phosphorylates and activates several transcription factors that support neuronal survival and learning and memory processes such as mTOR, cAMP response element-binding protein (CREB), the forkhead family of transcription factors, calcineurin, beta-catenin, c-Jun, Bad, p53 and the nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) [125]. In addition, Akt favors the synthesis of synaptic proteins by activating mTOR and the Akt/GSK3β/beta-catenin signaling pathway [126]. In addition, Akt regulates neuronal and dendritic morphology and its chronic inhibition induces dendritic retraction and reduction in soma size [127]. In neurons, activation of Akt is associated with elevation of axonal elongation and an increase of growth cone size [128]. It has been shown that chronic stress leads to a decrease of Akt activity and the synthesis of synaptic proteins in the brain of rodents. Thus, reduced Akt signaling may contribute to the neuronal atrophy, and synaptic and cognitive deficits induced by different brain disorders including PTSD [129] (Fig. 1). Furthermore, both typical and atypical antipsychotics stimulate Akt signaling, supporting the view that Akt signaling deficits may contribute to induce psychiatric symptoms. Inversely, the activation of Akt by cotinine induces beneficial effects on synaptic plasticity, neuronal survival, and learning and memory in other conditions leading to cognitive impairment. It has been shown that cotinine, in addition to the fear-conditioning model of PTSD, also stimulates Akt signaling and improved memory and mood in animal models of Alzheimer's disease [116, 118, 130–133], autism [119], schizophrenia, and chemotherapy-induced memory loss [122].

GSK3β is a protein kinase that is constitutively active and highly expressed in the brain, and which dysregulation has been proposed as a contributing factor in the etiology of several psychiatric disorders including PTSD [79, 119, 134]. GSK3β is tightly controlled by phosphorylation by neurotrophic factor receptors. GSK3β is inhibited by phosphorylation by protein kinase A and Akt in response to growth factors such as insulin and the BDNF [135]. It is thought that inhibition of GSK3 underlies the therapeutic effects of lithium in patients with bipolar disorder [134]. A recent paper showed a positive effect of cotinine improving memory in a mouse model of autism spectrum disorders [119]. Using a genetic approach, they demonstrated that the positive effects of cotinine on memory depended on the inhibition by phosphorylation of GSK3β by Akt at Ser 9 [119].

The Akt/GSK3β pathway is downstream of several receptors, including the α7nAChR, the tyrosine receptor kinase B/BDNF/NT-3 growth factor receptors, and the VEGFR, all considered potential therapeutic targets for neurological conditions [135]. Interestingly, cotinine stimulates cell signaling of both nAChRs and VEGFRs in the brain. In fact, cotinine stimulates the expression of VEGF in endothelial cells [136] and in the hippocampus of mice subjected to forced swim stress [137]. A detailed analysis of the potential role of these changes in VEGF expression on antidepressant and anti-Alzheimer's disease effects of cotinine have been previously discussed in detail [133, 138, 139].

Stimulation of the Nicotinic Receptors by Cotinine in PTSD

Cotinine is a nootropic compound [112] that is safe in humans [111, 140, 141]. The nAChRs are the most studied biological targets of cotinine and their positive modulation may easily explain the activation of Akt and its pro-cognitive effects. The nAChRs are ligand-gated ion channels that are broadly expressed in the CNS [142]. The low-affinity α7 and high-affinity α4β2 nAChRs are abundant in brain regions involved in cognition and mood regulation such as the amygdala, PFC, hippocampus, entorhinal cortex, hypothalamus, and striatum [143]. The activation of presynaptic and postsynaptic α7nAChRs promotes neurotransmitter release during brain development, and synaptic plasticity, memory, sensory gating, and attention in the adulthood [144, 145].

It is interesting that under stress conditions, nicotine stimulates the hypothalamus pituitary adrenal stress axis and consequently the release of corticosteroids and the expression nAChRs [146]. Paradoxically, this increase is accompanied by a decrease in the expression of the active form of the receptor, as reflected by the parallel inhibition of α-bungarotoxin binding to the α7nAChR [146]. In spite of its

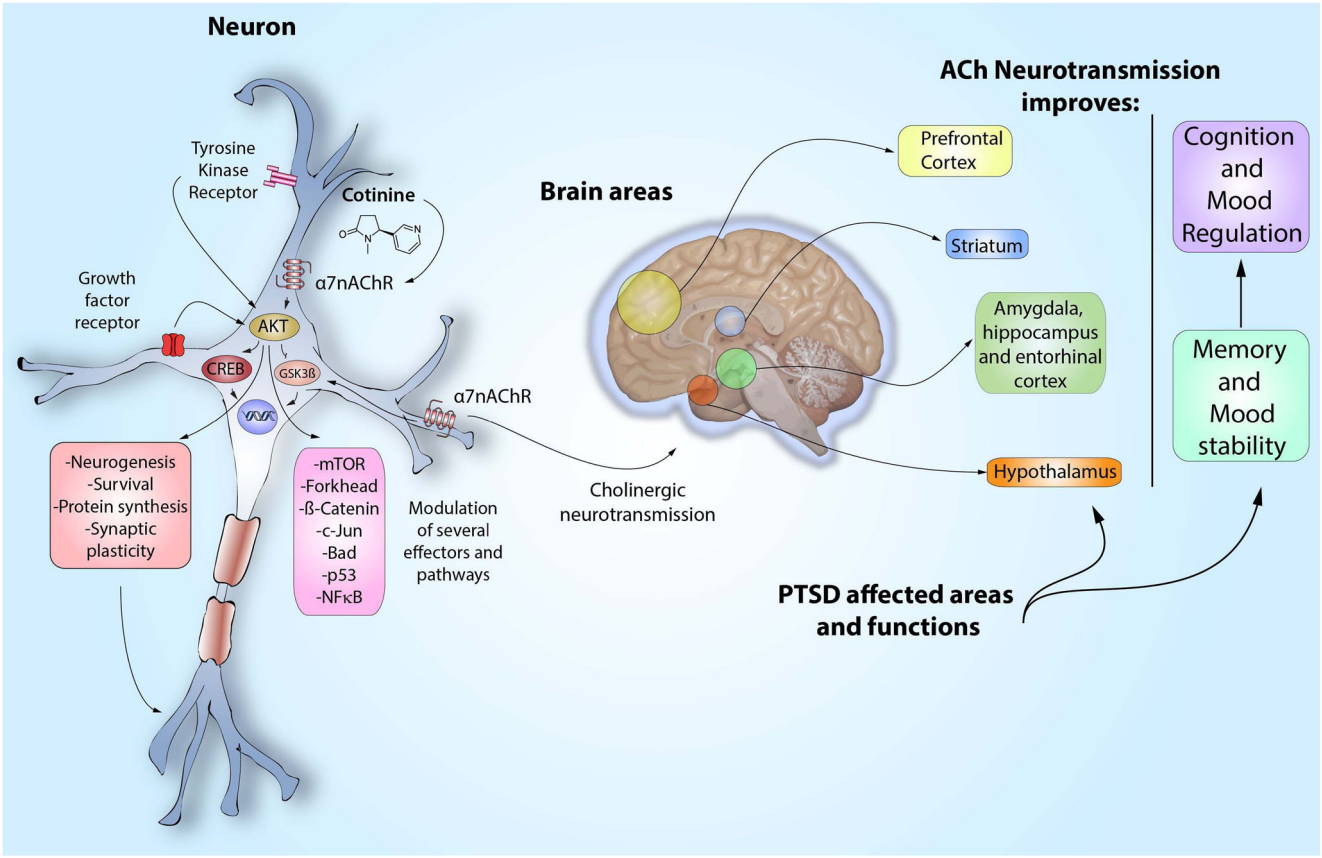

Fig. 1 Cotinine stimulates cell signaling pathways favoring synaptic plasticity. Cotinine is a natural alkaloid that improves neuronal functions in the brain. Molecular changes induced by cotinine include the activation of Akt/GSK3ß pathway with the ongoing transduction of mTOR, forkhead, Bad, p53, c-Jun and NFkB signals. Akt activation also includes the activation of CREB and CaMKK proteins. The modulation of Akt/GSK3ß pathway seems to be associated to nAChR—a nicotinic cholinergic receptor—expressed in critical brain areas as prefrontal cortex, striatum, amygdala, hippocampus, entorhinal cortex, and hypothalamus that control cognition, mood stability, and memory. The dysregulation of Akt/GSK3ß activation and signaling could be related with PTSD symptoms such as anxiety, depressive-like behavior, and fear

structural similarities, at difference with nicotine, cotinine is a poor agonist of the nAChRs [147, 148]. However, new evidence indicates that cotinine acts as a positive allosteric modulator, which binds to allosteric site(s) of the α7nAChRs, likely stabilizing the active form of the receptor at the plasma membrane of brain cells [149].

Several studies have shown evidence suggesting that cotinine induces its beneficial effects on brain function by acting on the nAChRs [107, 108, 150–154]. One of these studies investigated the effect of cotinine on DBA/2 mice having a deficit in hippocampal sensory inhibition. The CA3 regions of hippocampus were recorded to assess basal inhibition induced by paired identical auditory stimuli, then treated subcutaneously with cotinine, and recorded again. The results showed that cotinine at doses between 0.1 and 1.0 mg/kg induced a significant increase of the conditioning amplitude in mice. The effect was mediated by the nAChR since its antagonists, DHβE (α4β2) and α-bungarotoxin (α7), blocked the effect of cotinine [150]. The authors concluded that cotinine modulated sensory inhibition through the α4β2 and α7nAChR by acting as a positive allosteric modulator of these receptors.

Other studies showed that cotinine enhanced fear extinction in mouse [155] and rats [152]. One-week treatment with oral cotinine previous to fear conditioning (FC) did not prevent the increase in corticosteroid in plasma or induced basal changes in anxiety in the control or conditioned mice [155]. However, posttreatment with cotinine 1 day after fear conditioning reduced anxiety, depressive-like behavior, and enhanced fear extinction in the conditioned mice [155]. A similar positive effect on extinction was achieved with cotinine infused directly in the hippocampus of rats [152]. The effect of cotinine was prevented by the selective α7nAChR inhibitor methyllycaconitine (MLA) and the α4β2 antagonist dihydro-beta-erythroidine (DHβE).

The effect of the positive modulation of the nAChRs by cotinine can also depend on its effect in glial cells functions. Current evidence clearly indicates that neuroinflammation and changes in glial cells play important roles in the etiology and

development of psychiatric conditions such as major depression and PTSD [156, 157]. It is thought that microglia activation leads to neuroinflammation, oxidative stress, mitochondrial dysfunction, energy deficits, and neurodegeneration in mental diseases associated with aging and psychiatric conditions [158–163]. The positive modulation of the nAChR with cotinine and other similar compounds can reduce neuroinflammation [79, 164], as well as increase brain cell resilience to toxic injury by inhibiting microglia activation and the release of harmful cytokines such as the transforming necrotic factor alpha (TNFα) and IL-1 [110, 115, 165, 166]. Also, astrocyte numbers and complexity have been found affected in animal models of PTSD [14, 110, 115, 167]. Astrocytes support neuronal synaptic plasticity and energy balance as well as elicit neuroprotection against glutamate excitotoxicity by uptaking glutamate from the extracellular space [168–178]. In addition, it has been reported that neurons transfer damaged mitochondria to astrocytes that, in return, gave back functional mitochondria to neurons has been reported [179]. Thus, a positive effect over astroglia neuroprotective function can be central in the action of drugs for PTSD. Recently, we found a positive effect of intranasal post-treatment with cotinine on astrocytes numbers and complexity in a mouse model of immobilization stress. Further studies using other models of stress/depression/PTSD are currently investigated in our laboratory and futures developments in this regard are guaranteed.

Conclusions

Cotinine is an anxiolytic, antidepressant, and brain plasticity enhancer that promotes the extinction of contextual fear memory and it is safe in animals and humans. Latest evidence defined neurological and molecular mechanisms underlying cotinine's actions involving the pro-survival and pro-synaptic plasticity pathways such as the nAChRs/Akt/GSK3β, VEGFR/Akt/GSK3, and Akt/CREB/synaptophysin-PSD95 pathways. Furthermore, a positive effect of cotinine normalizing glial function under conditions of stress has been found. Based on this evidence, we propose that cotinine is an excellent choice to be tested as adjunctive therapy for PTSD, as well as other neurological and psychiatric conditions inducing neuroinflammation and learning and memory dysfunction.

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Artículo N°5 (enviado)

Cotinine plus Krill Oil decrease depressive behavior, and increased astrocytes survival in the hippocampus of mice subjected to restraint stress.

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Cotinine plus krill oil decrease depressive behavior, and increased astrocytes survival in the hippocampus of mice subjected to restraint stress

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Abstract

It has been shown that treatment with the alkaloid cotinine, prevented spatial working memory deficits and depressive-like behavior after prolonged restraint stress in mice. Furthermore, post-treatment with cotinine reduced the negative effects of restraint stress on astrocyte survival and arbor complexity. In this study, in the search for more effective combinations to be used with people subjected to immobilization or reduced mobility stress, it was investigated the effect of co-treatment with cotinine plus KO as a strategy to prevent depression and cognitive impairment and astrocyte modifications induced by restraint stress in the dental gyrus of mice. Our results show that both cotinine and cotinine plus KO have a glioprotective and neuroprotective effect diminishing the development of depressive-like behavior and recognition memory impairment induced by restraint stress. The use of the combination of cotinine plus KO to alleviate the deleterious effects of restraint stress (RS) in several medical conditions is discussed.

Key words: Depression; cotinine; anxiety; immobilization stress; krill oil; astrocytes.

Abbreviations: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid: AMPA; Analysis of variance: ANOVA; corticotrophin releasing hormone: CRH; Forced swimming: FS; Glial fibrillary acidic protein: GFAP; Hypothalamus-pituitary-adrenal: HPA; Immunoreactivity: IR; Krill oil: KO; Major depression: MD; Monoamine oxidase inhibitors: MAOIs; Mean gray values: MGV; Novel object recognition: NOR; Open field: OF; Phosphate buffered saline: PBS; Prefrontal cortex: PFC; Posttraumatic stress disorder: PTSD; Region of interest: ROI; Region serotonin-reuptake inhibitors: SSRIs; Serotonin norepinephrine reuptake inhibitors: SNRIs; Tris-buffered saline: TBS; TBS with 0.1% Tween 20: TBST.

1. Introduction

Stress induced by different noxious stimuli occurs when an individual is unable to cope with overwhelming physical or psychological demands. The ability to quickly change behavior and the underlying brain activity in response to threatening stimuli is crucial for survival. While acute stress can be beneficial in recruiting adaptive responses to cope with a stressful situation, prolonged stress can result in maladaptation that can be a risk factor for mental illness and both cognitive and motor deficits that further diminish the quality of life of people with restricted mobility.

Stress response although vital for survival when engaged for extended durations or activated by a traumatic event, it is linked to dysregulation of the HPA axis [1]. The dysregulation of the HPA axis correlates with altered levels of glucocorticoid hormones and neurotransmitters in the brain and the appearance of symptoms of depression [2]. The HPA axis has bidirectional relationships with the serotoninergic system. For example, amygdala neurons that are responsive to stress hormones such as the corticotrophin releasing hormone (CRH) project to the raphe nuclei, the main serotonin source to the forebrain, and projections from the raphe nuclei reach neurons containing CRH receptors in several brain regions controlling stress responses.

These pathological changes correlate with morphological alterations of the brain such as hippocampal volume reduction observed in individuals with major depressive disorder (MD) and/or posttraumatic stress disorder (PTSD)[3-15]. The deleterious effects of chronic stress include neuroinflammation, oxidative stress, microgliosis, decrease in neurogenesis, dendritic complexity of neurons and astrocytes, and number of synapses. These negative effects finally lead to working memory loss, impulsivity, aggressive behavior, and depression [16-20].

Chronic stress evokes synaptic alterations including spine loss and modulates spine shape, although, specific effects appear to vary across hippocampal sub-regions [21-23]. In rodents, immobilization stress affects both spatial memory [24, 25] and long-term potentiation in the hippocampus [26]. Such effects have been associated with the retraction of apical dendrites, as well as loss of synapses in the, CA1, CA3 and dentate gyrus sub-regions of the hippocampus [27, 28].

In recent years, numerous studies have highlighted the role of astrocytes in mediating brain homeostasis by supporting the blood-brain barrier function, sustaining neuronal energy metabolism [29] and neurotransmission [30]. Astrocytes also modulate synaptic plasticity processes including synaptic formation, neurogenesis, and learning and memory functions [31-33]. For these reasons, astrocytes are considered good therapeutic targets for several neurological disorders [34, 35].

Astrocytes are divided classified according to their cellular morphologies (cell body size, number of processes, thickness, direction, and length of processes) and anatomical locations into two main subtypes, protoplasmic or fibrous [36]. Protoplasmic astrocytes are found in gray matter and present numerous stem branches that originate several branching processes in a regular sphere-like distribution. Fibrous astrocytes are found in the white matter and have characteristic long processes [36]. It is believed that the morphological characteristics of astrocytes are important for their functions [37]. Recently, Choi et al. studied the molecular and morphological changes of astrocytes induced by fear conditioning. The results showed a significant decrease in the immunoreactivity (IR) for the astrocyte protein marker GFAP in the hippocampus of fear conditioned rodents [38]. Similar reduction in astrocytes in the CA1, CA3 and dental gyrus (DG) of the hippocampus was found in mice after prolonged restraint stress (RS)[39]. Previous studies indicated that cotinine induced consistent changes in all regions being most changes observed in the DG. In this study, we investigated the effect of the combination of KO plus cotinine on GFAP+ IR in the DG, selected as a representative zone of the hippocampus, in mice subjected to prolonged RS.

2. Materials and Methods

2.1. Animals

Two-month-old male C57BL/6J mice (obtained from the University of Chile), weighing 25-30 g were maintained on a 12-hours (h) light/dark cycle (light on at 07:00 h) with *ad libitum* access to food and water and at a regulated temperature (25 ± 1°C). Upon arrival, mice were group housed and acclimated for 7 days before behavioral testing. Experiments were

performed during the light period of the circadian cycle. Animal handling and care were performed according to protocols approved for the Universidad San Sebastian ethical committee and performed in compliance with the Guide for the care and use of laboratory animals adopted by the National Institute of Health (USA).

2.2. Drug preparation

Cotinine ((5*S*)-1-methyl-5-(3-pyridyl) pyrrolidin-2-one) (Sigma-Aldrich Corporation, St. Louis, MO, USA) was prepared by dissolving the powdered compound in sterile phosphate buffered saline (PBS). KO was purchased from Walgreens product KO omega-3, 300 mg capsules (Superba, USA). Soft gels contain 300 mg KO (omega-3 fatty acids 90 mg, eicosapentanoic acid 50 mg, docosahexaenoic acid 24 mg, phospholipids 130 mg). No information was provided by manufacturers about the Astaxanthin content in the soft gels.

2.3. Experimental groups and drug treatments

Mice after acclimatization and one week of handling were randomly divided into five groups (n = 8/condition) and orally treated as follow: 1) control non-restrained mice treated with vehicle (PBS, pH 7.4); 2) restrained mice treated with vehicle; 3), restrained mice treated with a cotinine solution (5 mg/kg in PBS, pH 7.4) via gavage; 4) restrained mice treated with KO (143 mg/kg); 5) restrained mice treated with cotinine plus KO solution. Mice were started with treatments, the first day of restraint and continuously until euthanasia. Treatments were administered at the same time of the day, 30 min before restraint. After 21 days into treatments, mice were behaviorally tested (Fig. 1).

2.4. Behavioral Procedures

2.4.1. Restraint stress

Restraint stress (RS) was used as a model of chronic stress-induced depressive-like behavior and cognitive impairment. We used this task because is a reliable method that mimics the effects of chronic stress without causing physical pain or unnecessary discomfort to the mice [40, 41]. Mice were gently introduced into a 50-ml conical transparent plastic tubes (Corning Inc.). The tubes contain non-protruding perforations in both ends and in the walls to permit ventilation and only permitted slight movements. Mice were kept inside these tubes at 25°C, during 6 h a day for 21 days. After the daily restraint time, mice were returned to their home cages and permitted to move freely for the rest of the day. Following the three weeks of RS, mice were behaviorally tested as described below.

2.4.2. Open field test (OF)

The open field (OF) test [42] was conducted as previously described with minor modifications [43] to identify changes in locomotor activity in response to stress and/or drug treatments. Mice were individually placed in a corner and permitted to freely explore an uncovered square arena (40 cm x 40 cm x 35 cm) for 25 min (Fig.2A). Total distance travelled, and time spent in the center zone were measured under moderate lighting using the video tracking software (ANY-Maze, Stoelting Co.).

2.4.3 Forced swim test

The forced swim (FS) is a broadly used task to assess depressive-like behavior in rodents [44]. The FS is performed introducing each mouse in the surface of a transparent and inescapable cylinder two-thirds filled with water at 26 ± 1 °C (Fig.3A). Mice engage in periods of intense movement followed for increasing periods of immobility. The immobility time during a 5-min trial is considered an expression of depressive-like behavior. Immobility time is defined as no longer exhibiting any escape behavior, motionless or moving only to

keep floating. Immobility time was recorded and quantified by two investigators blind to the treatment groups.

2.4.4. Novel object recognition (NOR)

This task evaluates recognition memory and it is based on the natural preference of rodents for novel objects when exposed to new and previously encountered objects [45]. During the task, favored exploration of the novel object provides a measure of recognition memory. After a habituation step in a square arena (40 cm x 40 cm x 35 cm), each mouse was placed in the same arena but containing two identical transparent objects located equidistant to each other (familiarization phase) and led to explore the objects for 5 min (Fig. 4A). Then, mice were returned to their cages and permitted to rest for 30 min. After resting, mice were placed back in the arena containing one of the familiar objects and a new object (Fig.4B). The time exploring the two objects is recorded during 5 min. Exploratory behavior was recorded and the time of exploration of each object was normalized for animal activity by calculating the exploration index (EI) that corresponds to the time spent by the mouse exploring one of the equal objects or the new object/ total time spent exploring both objects x 100%. The behavioral recording and analysis was performed using the (ANY-Maze, Stoelting Co.).

2.5. Morphological analyses of astrocytes in the dentate gyrus

2.5.1. Brain tissue preparation

For the protein analyses, mice were euthanized, and brains removed. Each brain was divided into two hemispheres. The left hemisphere of brains was dissected out to collect the regions of interest and quickly frozen for later analyses. For the immunohistochemistry (IHC) and fluorescent IHC (F-IHC) analysis the right hemisphere of each mouse brain was placed in 4% paraformaldehyde in PBS pH 7.4 at 4°C for 24 h. The tissues were embedded in 2% agarose molds for vibratome sectioning. The region of interest was located using the Paxinos

Atlas as a reference (Franklin and Paxinos, 2001), and serial sections of 20 μm (n ≥ 2/ mouse) were collected using the Vibratome Leica VT1000S and placed on positively charged slides (Biocare Medical, Concord, CA).

2.5.2. Immunofluorescence and Confocal Microscopy

For the F-IHC, samples were washes 3 times for 7 min with Tris- buffered saline (TBS), pH 7.8. The primary antibody anti- GFAP (1:50, BioSB) was diluted in diluent buffer, containing TBS supplemented with 1% bovine serum albumin (BSA) and 0.2% Triton X-100, and incubated with the tissue sections overnight (ON) at 4°C. After 3 washes with TBS for 10 min, sections were incubated with the secondary antibody, Cy2-conjugated rabbit anti-mouse IgG (1:200, Jackson Immuno Research, Pennsylvania, USA) diluted in TBS containing 1% BSA for 2 h at room temperature (RT). The samples were counterstained with Hoechst (1:1000) and mounted with fluorescence mounting medium (Prolong, Invitrogen). Confocal z-stacks were acquired using a LSM 780 confocal microscope (Zeiss, Oberkochen, Germany), z-stacks were normalized to maintain a consistent signal intensity through the depth of the sample, confocal z-stack image series were superposed in maximum intensity projections by ImageJ (National Institute of Health, Bethesda, MA, USA) for the measurements.

2.5.3. Morphometric analysis and cell counting

In each image, a region of interest (ROI) that represented the dentate gyrus was determined using free-hand drawing. For each ROI, the mean gray values (MGV), representing the area fraction with immunoreactivity for GFAP, were measured. To measure the fluorescence intensity of GFAP immunostaining in the dentate gyrus, maximum intensity projections of confocal z-stacks acquired from sagittal brain sections were converted into 8-bit greyscale images with 256 scales (pixel intensity 0 corresponding to no signal and 255 to maximal signal) by ImageJ software. To calculate the area fraction of GFAP+, binary image was

converted using the threshold feature of ImageJ to keep IR area. The area of thresholded images were divided by the total area of the ROI. For the GFAP+ cell counting, cell to be counted must had at least half of the cell nucleus visible on the edge of the ROI and cells were not included in the analysis if they were adherent to blood vessel walls.

2.5.4. Statistical analysis

To analyze the group and treatment effects, differences of the means between groups were analyzed using one-way analysis of variance (ANOVA), and post hoc Dunnet´s test to assess difference significance between groups. Differences were considered significant with $P < 0.05$.

3. Theory

Co-treatment with an oral formulation of cotinine plus KO during restraint stress will prevent the deficits in astrocytes in the DG of the hippocampus and this effect will also prevent the depressive-like behavior and cognitive impairment induced by chronic restraint stress.

4. Results

4.1. Effect of krill oil and cotinine on locomotor activity

To determine changes in locomotor activity in the mice induced by co-treatments during immobilization stress an open field test was performed. A one-way ANOVA analysis revealed that in the restrained mice there were no statistically significant differences in distance travelled (a measure of locomotor activity) (Fig.2B) or speed (Fig.2C) between treatment groups. Similarly, no significant changes in locomotor activity were observed in the control non-stressed mice treatment groups (data not shown).

4.2. Effect of krill oil and cotinine on depressive-like behavior

To further investigate whether the anti-depressant effect of cotinine observed by pre-treatment with cotinine before restraint stress, the effect of cotinine during and after prolonged RS was measured. A two-way ANOVA analysis revealed a significant effect of chronic stress on the levels of depressive-like behavior ($F(1, 38) = 15.35$, $P = 0{,}0004$) expressed as a general increase in the time spent immobile in the forced swim test by the restrained mice. Also, this analysis revealed a significant effect of treatments on depressive-like behavior ($F(3, 38) = 5.23$, $P = 0.004$). A multiple comparison test showed no significant differences between restrained vehicle-treated mice and restrained mice treated either with cotinine ($P > 0.05$) or KO ($P > 0.05$) (Fig.2). that between the mice subjected to RS, the mice co-treated with KO plus cotinine showed significantly lower levels of immobility that vehicle-treated restrained mice ($P < 0.01$) (Fig.3B).

4.3. Effect of krill oil and cotinine on recognition memory

To determine whether the co-treatments during RS influence recognition memory mice were tested for new object preference in the novel object recognition test. Non-significant differences were found between non-stressed and restrained mice in the familiarization step of the task, with all mice explored the equal objects almost 50 % of the time no showing a preference for any of the objects (Fig.4C). However, one-way ANOVA analysis revealed significant differences between groups on recognition memory when mice were exposed to a new object in the arena ($F(4, 48) = 4.286$, $P = 0{,}0049$). A multiple comparison test showed significant differences between the control non-restrained mice and the restrained mice treated with vehicle when compared to mice treated with cotinine alone ($P < 0.05$) and KO alone ($P < 0.01$). However, mice treated with cotinine plus KO showed non-significant differences in preference for the new object with the control non-stressed mice ($P > 0.05$) (Fig.4D).

4.4. Morphological and cell viability analyses of astrocytes

4.4.1. Cell counting

Cell count analyses of GFAP+ immunoreactivity of dentate gyrus was performed in two sections per mouse. One-way ANOVA analysis of cell counting of sections revealed significant effects of treatments on the number of GFAP+ cells in the dentate gyrus ($F(7, 46) = 4{,}883$, $P = 0{,}0004$). A multiple comparison test revealed no significant effect of treatments between the control groups. Different results revealed the effects of treatments in the restrained mice. A significant reduction in cell density in the dentate gyrus region in the vehicle-treated restrained mice were observed when compared to control non-stressed mice ($P < 0{,}001$). No significant effect of KO-treatment was observed on cell counting compared to vehicle-treated restrained mice. On the other hand, a significant increase of cell density was observed in the cotinine-treated and KO plus cotinine-treated restrained mice when compared to vehicle-treated restrained mice ($P < 0{,}05$) (Fig. 5A and B).

4.4.2. Mean gray value

One-way ANOVA analyses of gray scale measurements were performed for GFAP+ cell in the dentate gyrus. The analysis shown significant effect of treatments in IR intensity in the dentate gyrus ($F(7,33) = 5.104$, $P = 0{,}0005$). A multiple comparison test revealed no significant effect of treatments on mean gray value in the non-stressed mice. However, a significant decrease of the mean gray value intensity was found in the vehicle-treated restrained mice group when compared to vehicle treated control mice ($P < 0{,}05$). No significant effect in IR intensity were revealed when the KO-treatment restrained mice were compared to vehicle-treated restrained mice. On the other hand, a significant increase of the IR intensity shown the cotinine-treated restrained mice when were compared to vehicle-treated restrained mice ($P < 0{,}01$). Similar than the cotinine-treated mice, there was a

significant increase of the IR intensity in the KO plus cotinine-treated restrained mice when were compared to vehicle-treated restrained mice ($P < 0,01$) (Fig.5C).

4.4.3. Area fraction

The analysis of the percent area fraction occupied by GFAP+ cells revealed significant effects of treatments in the dentate gyrus of the hippocampus ($F (7, 34) = 17.28$, $P < 0,0001$). A multiple comparison analysis showed that vehicle-treated restrained mice had a significant decrease of the GFAP+ area when were compared to control non-stressed and vehicle-treated mice ($P < 0,001$). No significant changes were observed when KO-treated and restrained mice were compared to vehicle-treated restrained mice ($P > 0.05$). Nevertheless, a significant increase in the GFAP+ fraction area was found in the cotinine-treated and KO plus cotinine-treated restrained mice in the dentate gyrus compared to vehicle-treated ($P < 0,001$) (Fig. 5D).

5. Discussion

Chronic immobilization or reduced mobility stress can result from obesity, paralysis induced by vascular events such as stroke, spinal cord injury, advanced age, and many neurodegenerative conditions such as arthrosis, and ataxia. These events result in depression and cognitive impairment in the affected individuals.

RS is a broadly used model of stress-induced depressive-like behavior [46]. Prolonged RS results in morphological changes in the brain such as retraction of processes in hippocampal neurons and astrocytes [27, 47], neuroinflammation [1, 48, 49], cognitive deficits [50-54] and depressive-like behavior in rodents [46, 55]. It has been shown that cotinine administered before and after RS, reduces depressive-like behavior, synaptic deficits, astrocyte alterations and cognitive impairment compared to vehicle-treated mice [39, 56, 57]. In this study, we aimed to investigate the effect of co-treatment with cotinine alone or combined with KO, during and after chronic RS, on the development of depressive-like behavior and cognitive

impairment induced by chronic stress in mice. RS provoked a decrease in recognition memory and depressive-like behavior in the mice, however, the combination of cotinine plus KO prevented the decrease in escape-oriented behavior in the forced swim test, and the loss of recognition memory in the novel recognition memory task. These results suggest that the mix potentiate the beneficial effects of both individual components in preserving mood stability and cognitive abilities under conditions of chronic immobilization stress.

It is well established that chronic stress induces a deficit in glutamatergic neurotransmission by mechanisms involving a decrease of NMDA (N-Methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in the postsynaptic site in the prefrontal cortex and the hippocampus, two brain regions that are fundamental for mediating declarative and working memory abilities. This reduction in the number of synaptic glutamate receptors induces a decrease in the activity of brain networks controlling stress behavior including the prefrontal cortex-amygdala and prefrontal cortex-hippocampus pathways. Some evidence suggests that loss of glutamate receptors in neurons of the prefrontal cortex after repeated stress in rats, it is due to increased ubiquitin–proteasome-dependent degradation of these receptors [58, 59]. Previous studies, using rodent models of chronic stress, found a reduced proliferation of glial progenitor cells, and a decrease of GFAP+ cells in several brain regions, including the hippocampus and prefrontal cortex in rats. In rats, glucocorticoids can diminish the expression of GFAP in the PFC, resulting in > 20% reduction in GFAP expression that was accompanied by a decrease of the GFAP mRNA [60]. In addition, chronic RS inhibits the glutamate uptake by astrocytes enhancing excitotoxicity and long-term depression [61]. Furthermore, some evidence show that rats exposed to early life stress have a decrease in astrocytes levels in the frontal cortex in adulthood, indicating a long-term effect of stress on glial cells development [62]. It is reasonable to propose that a deficit in astrocyte´s function plays a role in the higher susceptibility to PTSD in persons with previous history of child abuse.

We have previously found a protective effect of cotinine administered alone via intranasal, against astrocytes decrease induced by RS. In this study, we found that co-treatment of mice with cotinine plus KO prevented the decrease in the number and complexity of astrocytes in the hippocampus of mice subjected to RS. In this study, we observed a beneficial effect of

cotinine in and cotinine plus KO but not KO alone in preserving the number and arbor complexity of astrocytes under conditions of RS.

We have previously shown that in the absence of stress, long-term cotinine treatment for up to eight months did not induce significant differences in sensory motor abilities or anxiety in mice [63]. Alike these results, no significant changes in locomotor activity in the mice treated with cotinine, KO or cotinine plus KO and subjected to RS were found. Thus, the superior effect of the combination of cotinine plus KO increasing the escape-oriented behavior in the FS test, cannot be explained by an increase in locomotor activity induced by the mix.

It is appealing that comparable results were obtained in the behavioral parameters tested, with a more significant effect of the mix cotinine plus KO than the individual components in the mix. The connection between changes in astrocytes and depressive-like behavior has been reported before. For example, a previous study reported that the diminution of astrocytes in the frontal cortex by using L-alpha-aminoadipic acid induced depressive-like behavior in rodents [64]. This evidence demonstrated that astroglia ablation in the PFC is sufficient to prompt depressive-like behaviors alike the one induced by chronic stress. This data strongly suggests that loss of astroglia may be a key factor contributing to the development of long-lasting depression [64].

The effect of cotinine in the mix preventing the effect of stress on mood can be the result of the action of cotinine as an anti-inflammatory compound inhibiting microgliosis and neuroinflammation as well as promoting neuronal and astrocyte survival throughout the activation of pro-survival cell signaling pathways.

Increased levels of astrocytes will provide neurons with more energy substrates, glutamate precursors and neurotrophic factors. In addition, astrocytes can decrease the toxic effect of the abnormal increase in glutamate release induced by corticosteroids at the presynaptic level, by uptaking the glutamate from the synaptic space. On the other hand, KO components such as omega-3 and Astaxanthin can prevent oxidative stress and diminish the deleterious effects of stress on brain function [65, 66](Fig.6).

A more detailed study of the effect on cotinine and KO on astrocyte function is guarantee at the light of the present results.

6. Conclusions

In this work it was investigated whether the mix cotinine plus KO administered as an oral formulation could be useful to prevent the cognitive and affect disturbances induced by chronic restraint stress. The results show that the mix at the doses tested, prevented the depressive-like behavior, memory impairment and astrocytes disturbances induced by RS and suggests that this formulation may be useful in people and animals subjected to restraint stress due to aging and pathological and traumatic conditions. Clinical studies are needed to confirm these results in humans.

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Declaration of conflict of interest

VE is the inventor of the patent (US 20100104504), for the use of cotinine for post-traumatic stress disorder. The other authors do not have conflict of interest.

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Figure Legends

Fig.1. Experimental design. Mice were subjected to restraint stress 6 h/ day for 21 days and co-treated with PBS, krill oil (KO), Cotinine (Cot) or Cot plus KO. After restraint and under continue treatments, mice were tested for locomotor function, recognition memory using the novel object recognition test (NOR) and depressive-like behavior using the forced swim (FS) test.

Fig.2. **Co-treatment with cotinine and krill oil does not affects locomotor activity in mice**. After prolonged restraint stress (RS) and co-treatment with vehicle (PBS), cotinine (Cot, 5 mg/kg), krill oil (KO, 143 mg/kg) or (Cot plus KO), mice were tested for locomotor activity in the open field test for 25 min. The results show that treatments did not affect locomotor activity in the mice. A, Total distance travelled. B, Mean speed (meters/seconds). Ns, non-significant difference ($P > 0.05$). ** significant difference ($P < 0.01$).

Fig.3. Co-treatment with cotinine plus krill oil prevented the restraint stress-induced depressive-like behavior in mice. Mice were tested for depressive-like behavior: A; Drawing representing the forced swim test (for details please review "Materials and Methods" section); B, after three-week restraint and co-treatment with vehicle (PBS),

cotinine (Cot, 5 mg/kg) or krill oil (KO, 143 mg/kg), mice were tested for depressive-like behavior in the forced swim tests (5 min).

Fig.4. Co-treatment with cotinine decreased the restraint stress-induced deficit in recognition memory. After restraint and co-treatment with vehicle (PBS), cotinine (Cot, 5 mg/kg) krill oil (KO, 143 mg/kg) or Cot plus KO, mice were tested for locomotor activity in the open field test and next day mice were tested for recognition memory in the novel object recognition test (NOR). A, Familiarization: mice were individually exposed to two identical objects. B, Novel object recognition step: after 30 min of rest, mice were exposed to one of the old objects and a new object. Chronic restraint stress impaired novel object recognition. Co-treatment with KO plus Cot preserved recognition memory abilities in the stressed mice to levels non-significantly different from control non-stressed mice ($p > 0.05$).

Fig.5. Analysis of the effect of cotinine plus krill oil on astrocytes in the dentate gyrus of the hippocampus. Figure representing the changes in cell GFAP+ cells numbers and morphology in the dentate gyrus region of the hippocampus in male mice subjected or not to restraint stress (R. Stress) (A). Graph depicting the changes in GFAP+ cells numbers (B); main grey values (MGV) (C); and area of immunoreactivity to GFAP (D), in the dentate gyrus of control mice or restrained (RS) mice treated with phosphate buffered saline (PBS), cotinine (Cot, 5 mg/kg) or krill oil (143 mg/kg) plus Cot (KO + Cot).

Fig.6. Diagram representing the effect of cotinine and krill oil preventing the effects of chronic stress on astrocyte and neuronal function and behavior. The mix cotinine plus KO may counteract the neuroinflammatory and oxidative processes triggered by chronic stress in the brain. This protection may prevent the astrocyte reduction in numbers and functions including the support of neuronal plasticity including neurogenesis and that is required for memory and mood stability.

Fig.1

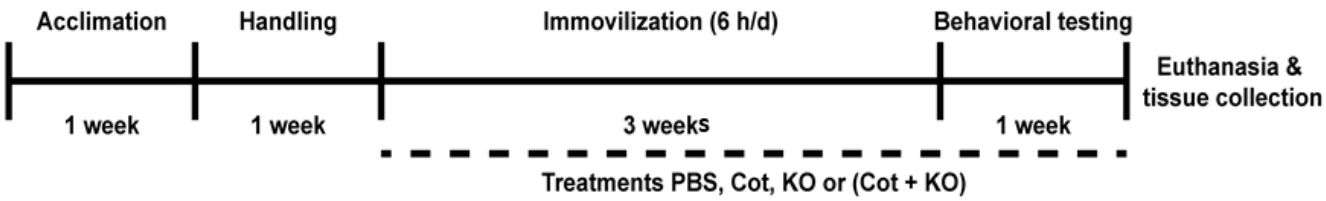

Acclimation
Handling
Immovilization (6 h/d)
Behavioral testing
Euthanasia &
tissue collection
1 week
1 week
3 weeks
1 week
Treatments PBS, Cot, KO or (Cot + KO)

Fig.2

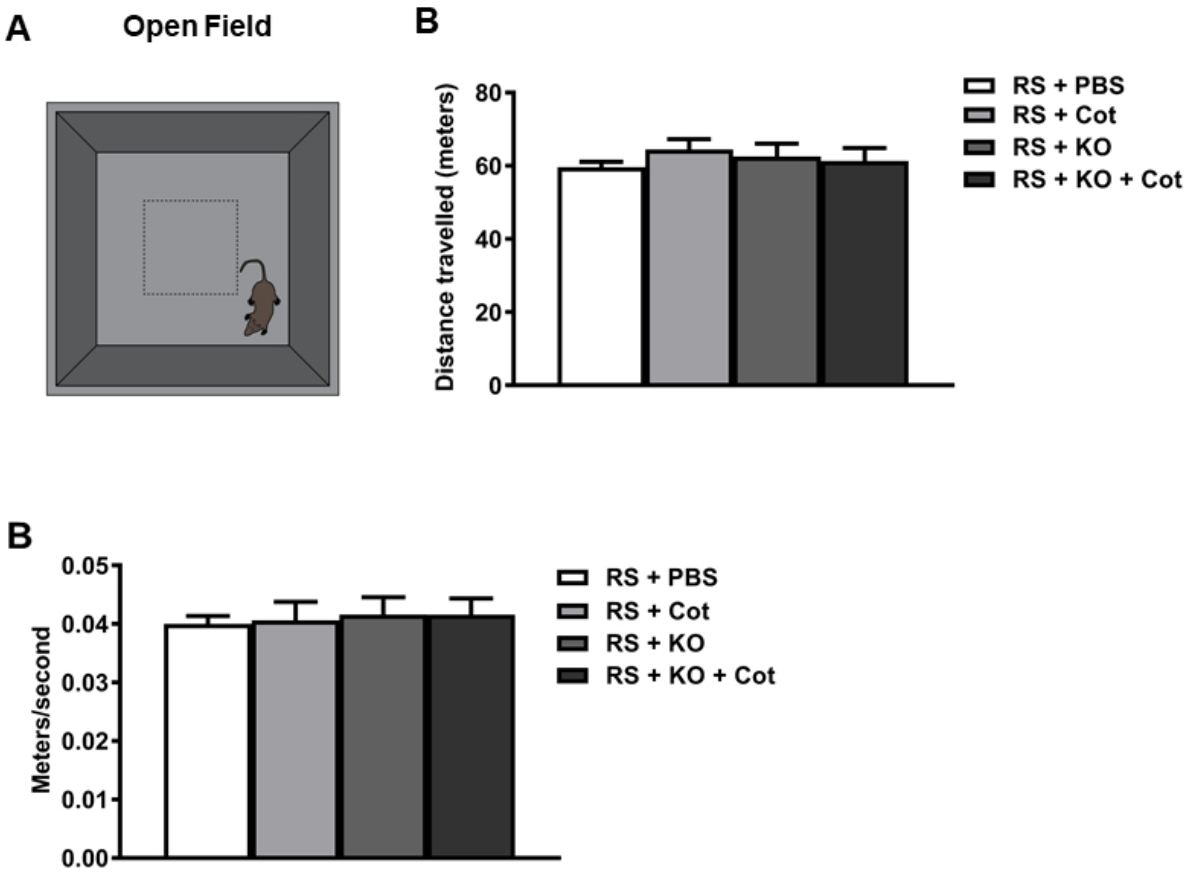

A
Open Field
B
Distance travelled (meters)
80
60
40
20
0
RS + PBS
RS + Cot
RS + KO
RS + KO + Cot
B
Meters/second
0.05
0.04
0.03
0.02
0.01
0.00
RS + PBS
RS + Cot
RS + KO
RS + KO + Cot

Fig.3

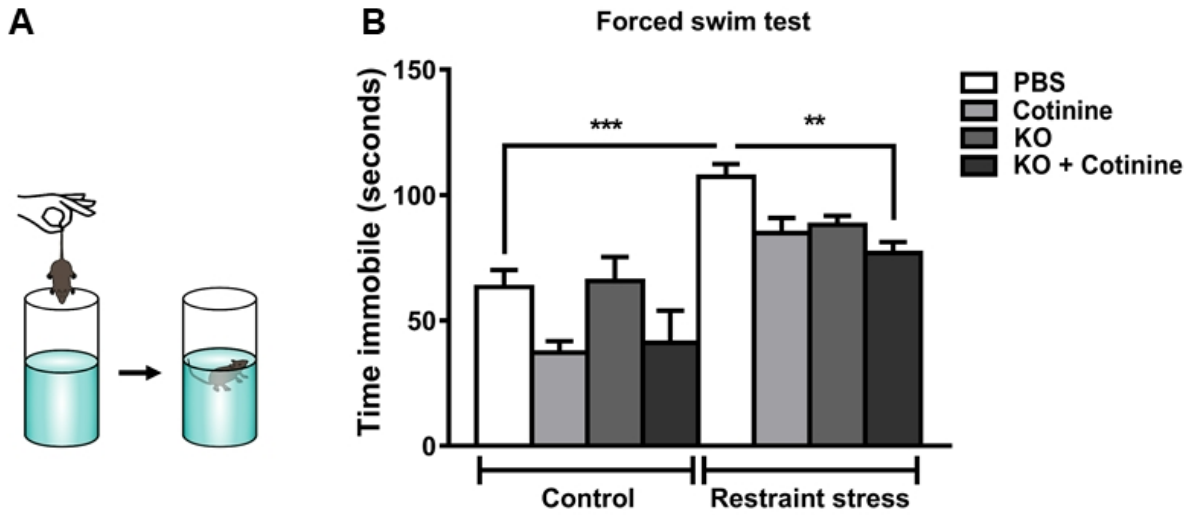

A
B
Forced swim test
Time immobile (seconds)
150
100
50
0
***
**
PBS
Cotinine
KO
KO + Cotinine
Control
Restraint stress

Fig.4

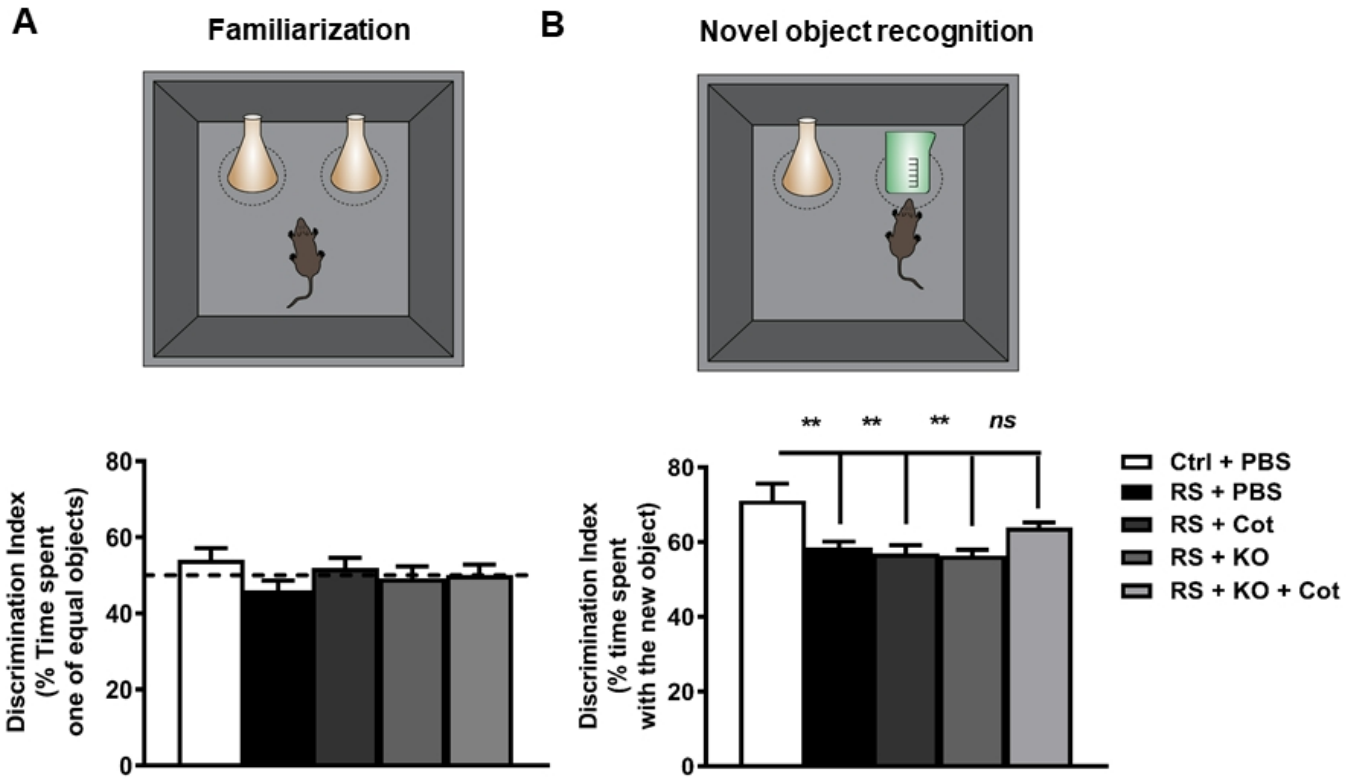

A
Familiarization
B
Novel object recognition
Discrimination Index
(% Time spent
one of equal objects)
Discrimination Index
(% time spent
with the new object)
80
60
40
20
0
** ** ** ns
Ctrl + PBS
RS + PBS
RS + Cot
RS + KO
RS + KO + Cot

Fig.5

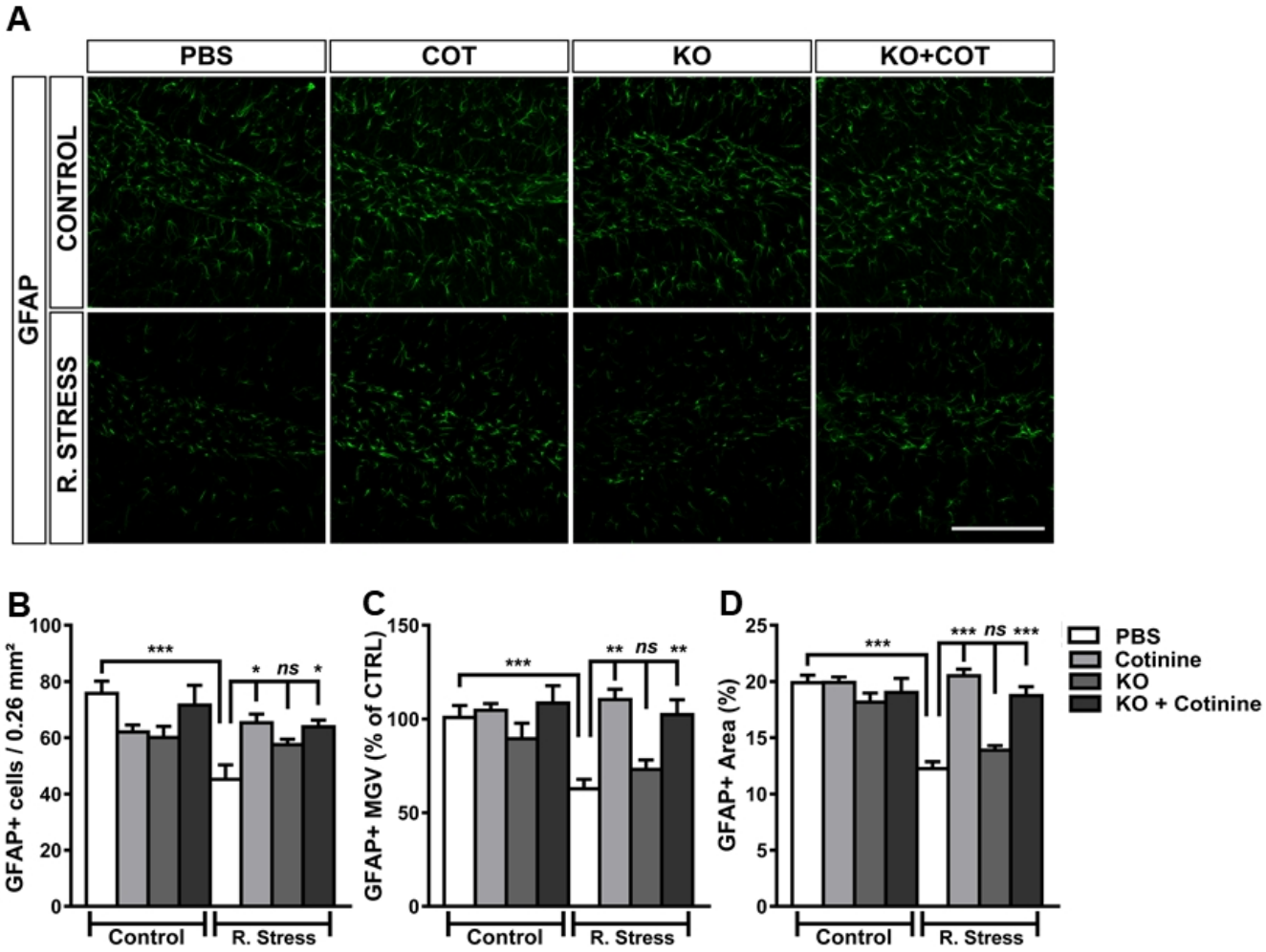
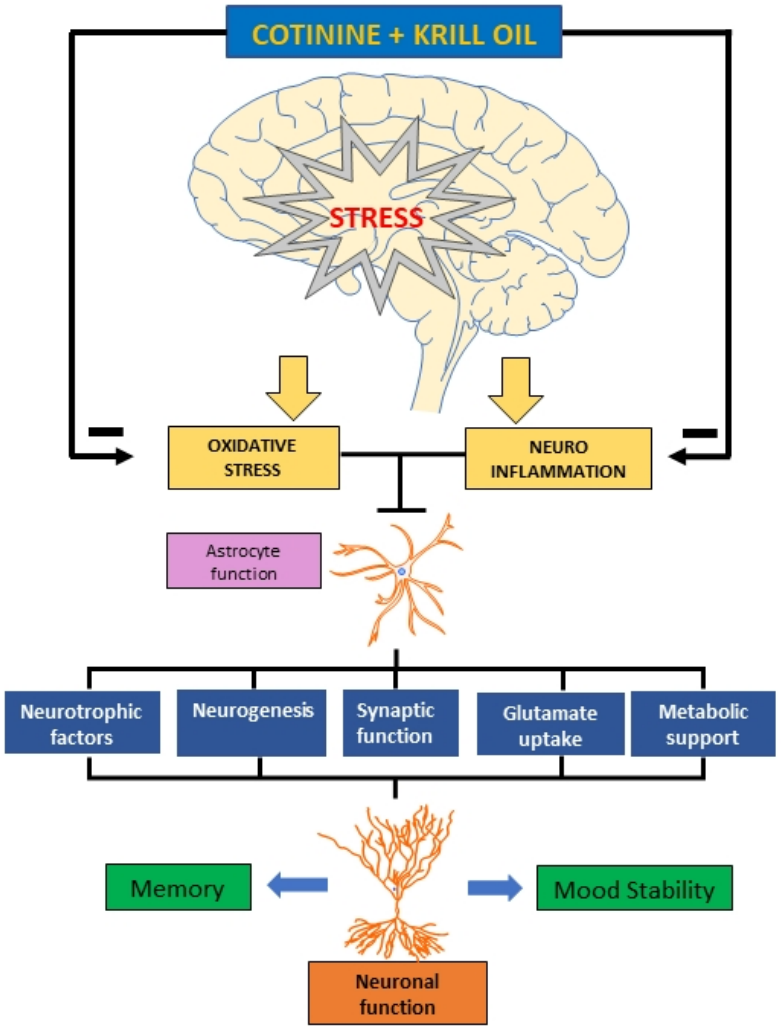

A
PBS
COT
KO
KO+COT
GFAP
CONTROL
R. STRESS
B
GFAP+ cells / 0.26 mm²
100
80
60
40
20
0
***
* ns *
Control
R. Stress
C
GFAP+ MGV (% of CTRL)
150
100
50
0
***
** ns **
Control
R. Stress
D
GFAP+ Area (%)
25
20
15
10
5
0
*** ns ***
Control
R. Stress
PBS
Cotinine
KO
KO + Cotinine

Fig.6


COTININE + KRILL OIL
STRESS
OXIDATIVE STRESS
NEURO INFLAMMATION
Astrocyte function
Neurotrophic factors
Neurogenesis
Synaptic function
Glutamate uptake
Metabolic support
Memory
Mood Stability
Neuronal function

VI. CONCLUSIONES

Artículo N°1

- En conjunto, la evidencia actual respalda el importante papel de la inflamación y las hormonas sexuales en los aspectos conductuales y neurofuncionales del PTSD.
- Las diferencias de género en la prevalencia de los trastornos de estrés pueden explicarse por una modulación diferencial de las hormonas sexuales del sistema inmune y las respuestas cerebrales al estrés en sujetos masculinos y femeninos.
- Sin embargo, en la complejidad del PTSD, la edad y otros factores ambientales y culturales pueden influir, en gran medida, en el efecto de las hormonas del estrés sobre el desarrollo del PTSD.
- Se requieren más estudios, correlacionando las hormonas sexuales, el género y las influencias ambientales en el desarrollo del trastorno de estrés postraumático. Una mejor comprensión del papel de estos factores puede llevarnos a terapias preventivas o curativas más efectivas.

Artículo N°2

- La evidencia obtenida en este estudio permite concluir que el posttratamiento con cotinina (administración Intranasal, IN) es eficaz para restablecer el equilibrio del estado de ánimo y las capacidades cognitivas, así como la función de los astrocitos después del estrés de restricción crónica en ratones.
- Lo anterior constituye la primera evidencia sobre la acción de la cotinina sobre las células GFAP +. Este hallazgo representa un nuevo mecanismo de acción de la cotinina para restaurar la supervivencia neuronal y la plasticidad después del estrés.
- El suministro IN (de cotinina) demostró ser eficaz como un método de tratamiento con cotinina para el trastorno de estrés postraumático o trastornos asociados con el estrés por restricción.
- Es necesario complementar los resultados presentados en este trabajo con más investigaciones clínicas, lo que permitirá establecer si los efectos beneficiosos observados de la cotinina en roedores son igualmente efectivos en humanos.

Artículo N°3

- Los resultados sugieren que el tratamiento a corto plazo con cotinina intranasal más aceite de krill es mejor que la sertralina y el aceite de krill solo para aumentar la extinción del miedo. A pesar de que el aceite de krill más la cotinina es sólo ligeramente superior a la cotinina sola en disminuir la respuesta de miedo, el uso de la mezcla tiene la ventaja adicional de que el aceite de krill tiene efectos beneficiosos sobre la salud vascular.
- Se necesitarán más estudios clínicos para confirmar completamente el valor terapéutico de la cotinina intranasal sola y en combinación con el aceite de krill para facilitar la recuperación de personas con trastorno de estrés postraumático.
- La evidencia muestra que la cotinina intranasal sola o en combinación con aceite de krill facilita la extinción de la memoria de miedo contextual y disminuye el comportamiento depresivo, a una dosis 10 veces menor que la dosis oral previamente activa de cotinina en ratones.
- Los efectos pro colinérgicos, antioxidantes y antiinflamatorios de ambos compuestos pueden explicar sus efectos sinérgicos positivos sobre la depresión.
- El efecto de la cotinina en la calcineurina A parece ser otro mecanismo de acción crítico de la cotinina contra la patología del PTSD, pero el tema requiere una investigación más profunda para demostrar una relación causal directa con los efectos beneficiosos de la cotinina.
- En general, esta evidencia preclínica respalda la investigación clínica de cotinina intranasal más aceite de krill para reducir los síntomas depresivos derivados de recuerdos traumáticos asociativos, recurrentes en pacientes con PTSD.

Artículo N°4

- La cotinina es un ansiolítico, antidepresivo, y potenciador de la plasticidad cerebral, que promueve la extinción de la memoria contextual del miedo y es segura en animales y humanos.
- La evidencia más reciente definió los mecanismos neurológicos y moleculares subyacentes a las acciones de cotinina que involucran las vías de pro-supervivencia y plasticidad pro-sináptica, como las vías nAChRs / Akt / GSK3β, VEGFR / Akt / GSK3 y Akt / CREB / sinaptofisina-PSD95.
- Además, se ha encontrado un efecto positivo de cotinina que normaliza la función glial en condiciones de estrés.

- Con base en esta evidencia, proponemos que la cotinina es una excelente opción para probarse como terapia adyuvante para el PTSD, así como otras afecciones neurológicas y psiquiátricas que inducen neuroinflamación y disfunción del aprendizaje y la memoria.

Artículo N°5 (Enviado)

- En este trabajo se investigó si la mezcla de cotinina más aceite de Krill administrada como una formulación oral (cotratamiento) podría ser útil para prevenir las alteraciones cognitivas y afectar las perturbaciones inducidas por la restricción crónica.
- Los resultados muestran que la mezcla, a las dosis probadas, evitó el comportamiento depresivo, la alteración de la memoria y los trastornos en los astrocitos, inducidos por la restricción del movimiento.
- Se sugiere que esta formulación puede ser útil en personas y animales sometidos a estrés por restricción, debido al envejecimiento, condiciones patológicas o traumáticas.
- Se necesitan estudios clínicos para confirmar estos resultados en humanos.